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Production and Biochemical Characterization of Fermented Sorghum

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Production and Biochemical Characterization of Fermented Sorghum

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Abstract

Sorghum (*Sorghum bicolor*) is one of the super crops and the fifth most important cereal with high nutrient value. It is gluten free cereal with high content of various phenolic compounds among which phenolic acids, flavonoids and condensed tannins are main compounds. The goal of this study is to increase the L-carnitine concentration as well as other functional components of sorghum by fermentation. Fermented samples were prepared for morphological study like SEM, analytical study like LC/MS (L-carnitine, citrinin and phenolic compounds), biochemical study like TPC, TFC, TTC, DPPH, FRAP and ORAC. Degradation on the outer surface of sorghum observed after fermentation by *Monascus purpureus* K/M where non-fermented sorghum have smooth surface. L-carnitine amount was increased from 0.42±0.05 mg/kg to 120.6±0.7 mg/kg after 14 days fermentation by *M. purpureus* K/M. The total phenolic and total flavonoids contents were increased during fermentation. On the other hand, total tannin contents were decreased to 290% as the fermentation day increased. DPPH, FRAP and ORAC assays showed the increase of antioxidant activities compared to non-fermented sorghum. Gallic acid and vanillic acid were appeared after fermentation. The inhibitory activity of α- glucosidase and mushroom tyrosinase also increase 199.6% and 141.4% after 5 days fermentation. After fermentation by *M. purpureus* K/M, the l-carnitine content in sorghum increased 287 times. The cell toxicity of fermented sorghum was decreased

compare to non-fermented sorghum. 76.9%, 74.6% and 77.6% inhibition of NO production on macrophage cells were observed in non-fermented, 5 days and 7 days fermented sorghum, respectively Based on these findings, fermented sorghum has potential application as functional materials in nutraceutical, pharmaceutical, and cosmeceutical industries.

Keywords: Sorghum, Fermentation, L-carnitine, Anti-inflammatory activity, *Monascus purpureus*.

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Research background

1. Sorghum

Sorghum (*Sorghum bicolor* L.) was cultivated in North eastern Africa 3,700 to 4,000 years ago and one of the fifth highest producing cereal crop next to maize, wheat, rice and barley[1]. Recently, sorghum is gaining attention because of its gluten free status, decent portion of resistant starch as well as high amount of diverse bioactive phytochemicals compared to other major cereal crops[2]. These phytochemicals often related with prevention of the risk of type II diabetes, cardiovascular diseases, oxidative stress, cancer and help with weight loss[3-5].

Fig 1 *Sorghum bicolor*

2. Source and importance of L-carnitine

Lysine and methionine used as substrates for synthesis of L-carnitine which is one of the non-essential amino acid[6], play major role in energy production by transporting long chain fatty acid into the mitochondrial compartment for β-oxidation[7]. Not only in energy production, L-carnitine also helps in weight loss[8], improves bone and heart health[9] and can induce apoptosis of certain carcinoma cells[10] as well as have neuroprotective activity that can use in the treatment of alzheimer's disease[11]. Human body can produce approximately 1-2 μmol of carnitine/kg[12] in one day despite the majority of L-carnitine is acquired by consumption of meat, fish, poultry or dairy produce which are the main source of L-carnitine[13]. People with cereal-based diet have lower plasma carnitine compare to the people with animal-based diet as vegetable, grain, legume and fruit contain limited amount of L-carnitine[14-16].

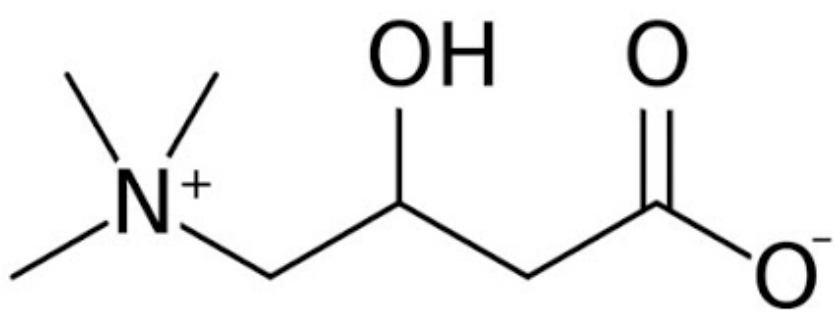

Fig 2 L-carnitine structure (Source: quimicaalkano.com)

3. Biochemical improvement of sorghum by solid state fermentation

Although sorghum is rich in many health promoting phytochemicals, the use of sorghum as staple food is restricted due to anti-nutritional factors like tannin and phytic acid which lower bioavailability of nutrients in sorghum[17]. Hence, it is necessary to process sorghum grain using methods like soaking, thermal or enzymatic treatment to lower these anti-nutritional compounds and increase the physiochemical properties to produce sorghum grain based food and feed[18-19]. Solid-state fermentation (SSF) by using filamentous fungi is another alternative treatment for sorghum processing. It also improves the nutritional properties by increasing essential amino acids content, phenol content, flavonoid content, antioxidant capacity and by decreasing anti-nutritional factors[20-21].

4. Edible fungus used in fermentation

Rhizopus oligosporus and *Monascus spp.* are mostly used for the production of many fermented foods by solid-state fermentation. *R. oligosporus* is a GRAS fungus declared by USDA as it does not produce any mycotoxin and is a dominant microbial species in the fermented foods like tempeh which is a traditional food in Indonesia[22]. Several studies showed that after fermentation with *R. oligosporus* GABA, antioxidant and phenolic contents increase[23-25]. As *R. oligosporus* possess enzymes like β-glucosidase, glucosidase, glucuronidase and xylanase which can hydrolyse carbohydrates-bound phenolic compounds and increase free phenolic acids and flavonoids[20].

5. *Monascus* spp.

For many centuries, Asian countries use *Monascus spp.* for the production of *Monascus* fermented rice commonly known as red yeast rice. *Monascus spp* well known for the production of pharmacologically active compounds like monacolins, GABA, monacsin, dimerumic acid and pigments[26]. Monacolin K is one of the derivatives of monacolin produced during fermentation can inhibit a key enzyme responsible for cholesterol synthesis called 3-hydroxy-3-methylglutaryl-coenzyme A reductase[27]. *Monascus spp.* pigments are used as food colorant, preservative and in medicine and have anti-obesity, anti-cancer, anti-microbial, anti-inflammation activity[28-29]. In addition, *Monascus spp*. also improves antioxidant activity and reduces anti-nutrient after

fermentation[30-31].

6. Antidiabetic activity

Type 2 diabetes is one of the chronic and uprising disease mainly caused by insulin resistance[32]. One of the way to control the disease is to inhibit of carbohydrate degrading enzyme like α-glucosidase to lower the blood glucose level[33]. Synthetic inhibitor like acarbose is available but it can cause digestion problem like bowel irritation as well as have other side effects[34]. So now a day's researchers are focused on finding natural inhibitors especially plant derivatives. Sorghum is rich in phenolic compounds which possess inhibitory ability against α-glucosidase[35]. Previous study showed *Monascus* fermented products have antidiabetic activity which is positively related with the rising amount of phenolic compound during fermentation[36].

7. Antityrosinase activity

Now a days many cosmetic industry try to develop products using natural bioactive compounds with high antioxidant activity to inhibit the tyrosinase which become highly active by overexpression of protease enzyme and cause pigmentation[37-38]. Phenolic acids and peptides extracted from sorghum as well as *M. purpureus* fermented rice bran have inhibition activity against tyrosinase enzyme[39-40].

8. Research objectives

The purpose of this study is to increase the production of L-carnitine in sorghum through fermentation using *M. purpureus* K/M, as well as to enhance the phenolics, flavonoids and biological properties such as antioxidant, antidiabetic, antityrosinase, and anti-inflammatory activities for development of functional food materials. Previous study reported that, L-carnitine in *R. oligosporus* fermented buckwheat and quinoa increased four times compare to the non-fermented one[24-25]. Studies about *Monascus* spp. fermented sorghum have been reported but they focused on nutritional quality, production of monacolin-k and pigment[41-42]. There is no report about production of L-carnitine by *M. purpureus* K/M. This study is the first to report the production and enhancement of L-carnitine by *M. purpureus* K/M by fermentation.

Materials and methods

1. Materials

Red sorghum was purchased from Achim Agricultural multigrain co., Ltd (Korea) and stored at 4º C. L-carnitine standard was brought from TCI (Seoul, Korea). Catechin hydrate, gallic acid, ferulic acid, chlorogenic acid, p-coumaric acid, vanillic acid, caffeic acid, protocatechuic acid, tannin as well as 2, 2-diphenyl-1-picrylhydrazyl, Folin–Ciocalteu reagent and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid were also obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade ethyl alcohol and acetonitrile were acquired from Honeywell—Burdick and Jackson (Muskegon, MI, USA). Formic acid was purchased from Thermo Fisher Scientific Korea LTD (Seoul, Korea) and fluorescein was obtained from Alfa Aesar (Haverhill, MA, USA).

2. Culture condition of microbial strain

Monascus purpureus K/M was isolated and deposited KCCM (Seoul, Korea). This fungal was inoculated on Potato Dextrose Agar (PDA, Difco, Maryland, USA) medium for 10 days at 30 ºC.

3. Preparation of fermented sorghum

Fifty gram of red sorghum was dunked in 50 mL distilled water for 10 h and autoclaved for 15 min at 121 °C. For spore collection, *M. purpureus* K/M was cultured on PDA plates at 30 °C 10 days[43] then 5×10^4 spores was inoculated to per gram sterilized sorghum and incubated at 30 °C for 3–14 days. At -80 ºC the fermented sorghum was frozen and freeze dried at − 10 to 0 °C under 20 Pa (Tokyo Rikakikai Co., Tokyo, Japan). Lyophilized sorghum was stored in dark condition at -20 °C for further study.

4. Extraction of sample

Two gram of lyophilized fermented and non-fermented sorghum were sonicated at 30 s interval, 40 μm amplitude for 10 min using ultra sonication processor (Vibra cell^{TM} Sonics, Newtown, USA) in 20 mL of 70% ethanol. To increase the extraction yield, the sorghum samples were further sonicated at 40 °C, 230 V; 60 Hz for 60 min in ultra sonicator water bath (WiseClean, Daihan scientific Co. Ltd, Wonju, Korea) and this process was repeated three times. Then the extracted samples were centrifuged at 12,000 × g for 15 min and filtered through paper filter (Whatman, Piscataway, NJ, USA). Ethanol was evaporated (Heidolph Instruments, Schwabach, Germany) and the extracted samples were freeze-dried. The extraction yield was calculated by the following equation;

$$\text{Yield (\%)} = \frac{\text{Extract mass (g)}}{\text{Sorghum powder mass (g)}} \times 100$$

5. Scanning electron microscopy (SEM) observation

Scanning electron microscope (SEM, TM 3030plus, Hitachi, Tokyo, Japan) was used to observed the change on the surface of sorghum before and after fermentation. In secondary electron (SE) mode at magnification of 50×, 5.0 kV of accelerate voltage the fermented and non-fermented whole sorghum grains were observed. After removal of fungal hypha, grain surface images were taken at magnification of 100× and 200 in secondary electron (SE) mode.

6. L-carnitine content analysis

L-carnitine amount in both control and fermented sorghum extracts were analysed by our earlier study[43]. In 1 mL of dimethyl sulfoxide (DMSO), 10 mg of sample was dissolved by vortexing for 60 min. After the sample was completely dissolved it was diluted to 1 mg within 1 mL HPLC grade methanol, centrifuged at 12,000 × g for 10 min and filtered with 0.2 µm filter (Satorius AG, Goettingen, Germany). One µL of sample was injected and analysed by UPLC/MS system (Waters, Milford, MA, USA) equipped with Waters Acquity H-Class system with Waters QDa detector. At 40 ºC, chromatographic separation was obtained on a Waters BEH HILIC column (1.7 µm × 2.1 mm × 100 mm). Mobile phase was consisted of two solvents; solvent A: aqueous formic acid 0.1% with acetonitrile and solvent B: formic acid 0.1% with 15 mM ammonium formate in distilled water at a flow rate of 0.4 mL/min. L-carnitine content was analysed by the following elusion gradient: initially 90% A, decreased to 70% A at 3 min, continuously decreased to 60% A at 5 min, back to initial gradient at 7.1 min and maintained till 10 min resulting in a total run time of 10 min[43]. 0.02 to 2 µg/mL of L-carnitine was used to develop an external standard method to quantify the L-carnitine in fermented sorghum and linearity between standards concentrations vs area was evaluated ($R^2 > 0.99$).

7. Citrinin analyses

Citrinin was determined in *Monascus purpurus* K/M fermented sorghum extracts according to the previous report[44] by slight modification using Waters Acquity H-Class system. Ten milligram of DMSO stock sample was diluted in methanol upto 1 mg/mL. One-milligram sample was injected and analyzed by UPLC/MS system. At 40 ºC, chromatographic separation was obtained on a BEH C_{18} column (1.7 µm × 2.1 mm × 100) and the mobile phase composed of two solvent system; solvent A: aqueous formic acid 0.1% with acetonitrile and solvent B: formic acid 0.1% in distilled water. The gradient elusion initiated with 40% B eventually decreased to 100% B at 6 min and back to initial gradient at 7 min and maintained till 10 min. To determine the citrinin in fermented sorghum, 0.001 to 2 µg/mL of citrinin was used to develop an external standard method and linearity was evaluated ($R^2 > 0.99$).

8. Phenolic compounds analyses

UPLC/MS analyses of phenolic compounds were carried out using Waters Acquity H-Class system with Waters QDa detector by Park (2017). Caffeic acid, ferulic acid, p-coumaric acid, gallic acid, vallic acid, protocatechiuc acid vanillin and catechin phenolic compound standards were dissolved in DMSO as 10 mg/mL and were diluted with methanol. Ten milligram of sample was dissolved by vortexing for 60 min in 1 mL of DMSO and diluted to 1mg in 1 mL methanol, centrifuged at 11,200 × g for 10 min and filtered. One microliter of sample was injected in LC/MS system and chromatography separation was achieved by using Waters BEH C_{18} column at 40 ºC. The mobile phase consisted of two solvent system; solvent A: formic acid 0.1% in 100% acetonitrile and solvent B: formic acid 0.1% in distilled water. Phenolic compoundss analyzed by the following elution gradient: initially 5% A, increased of A to 50% at 0.5 min, continuously increased of A to 70% at 1 min, 100% at 1and 2 min, decreased to 5% A from 2.10 to 4 min, 23% A at 10 min, 50% A at 12 min, 60% A at 15 min, from 15.10 to 16 min 100% A, back to initial gradient at 16.10 min and maintained till 25 min[43]. 0.02 to 2 μg/mL of different phenolic compounds were used to develop an external standard method and linearity between concentrations of standards vs area was evaluated ($R^2 > 0.99$).

9. Analysis of volatile compounds by using gas chromatography-mass spectrometry method (GC-SPME)

Volatile compounds in sorghum was analyzed by using GC (7890N, Agilent, NY, USA) equipped with a head-space sampler (7890N, Agilent, NY, USA)[45]. The detector was a mass detector (5975C MSD, Agilent, NY, USA). Volatile compounds in the sorghum were collected using solid phase micro extraction (SPME) fiber (50/30 μm DVB/CAR/PDMS). Two gram of sorghum powder were prepared in HS-GC vial and heated in the head-space sampler for 10 min at 60 ℃. Then, samples were extracted by GC-SPME fiber for 20 min and the temperature of injection port was set at 80 ℃. The temperature of the oven was maintained at 50 °C for 3 min, increased to 150 °C at a rate of 5 °C/min, 200 °C at a rate of 10 °C/min, 280 °C at a rate of 50 °C/min and remained unchanged for 3 min. The range of mass detection was 33 to 500 m/z.

10. Measurement of TPC

Investigation of total phenolic contents of fermented and non-fermented sorghum samples were done by using Folin ciocalteu's method[24] where gallic acid was used as the standard. The results of TPC was expressed as gallic acid equivalents in mg GAE/kg dry sample.

11. Measurement of TFC

Total flavonoid contents were analysed using aluminium chloride method[24] with minor modifications where quercetin used as a reference compound. Fermented and non-fermented sorghum extracts were prepared by dissolving in 1 mL of DMSO. Each sample was mixed with 60 µL of methanol and centrifuged at 11,200 × g for 10 min. Four microliter of 1 M potassium acetate, 4 µL of aluminium chloride and 112 µL of distilled water added, respectively. Then the mixture was incubated in dark condition at 28 ºC for 30 min at 415 nm wavelength absorbance was read by using SpectraMax. TFC were determined by quercetin equivalents in mg QE/kg dry sample.

12. Total condensed tannin content (TCT) analysis

For *Monascus purpureus* K/M fermented sorghum the tannin content was determined by Folin-Ciocalteu method[46] with some modification. Briefly, 120 µL of extracted sorghum was mixed with 15 µL of Folin-Ciocalteu reagent and shaked for 5 min. Fifteen microliter of 20% (w/v) sodium carbonate solution was added and incubate at 28 ºC for 30 min in dark condition. After 30 min absorbance was measured at 700 nm by using SpectraMax M3 [46]. Tannic acid standard at different concentration (3, 5, 10, 20, 30, 40, 50, 60, 90, 80, 100 µg/mL) was prepared and the condensed tannin was expressed as mg TA/kg dry sample.

13. DPPH radical scavenging activity

1,1-Diphenyl-2-Picrylhydrazyl radical scavenging activities were determined to estimate the antioxidant activities of non-fermented and fermented sorghum[47]. Ten milligram of extracted sorghum were dissolved in DMSO, diluted in ethanol and centrifuged at 11,200 × g for 10 min. One mM of DPPH was mixed thoroughly with sorghum extract and reacted for 30 min at 28 ºC in dark condition. The absorbance was checked at 517 nm by using SpectraMax M3. Radical scavenging activity was calculated as follows[48]:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{OD of control} - \text{OD of test sample}}{\text{OD of control}} \times 100$$

14. Ferric reducing antioxidant power (FRAP) assay

Antioxidant activity of sorghum samples were determined by FRAP assay [49] with some modifications[50]. The working solution was prepared by using 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) dissolved in 40 mM HCl, 300 mM sodium acetate buffer at pH 3.6 and 20 mM $FeCl_3 \cdot 6H_2O$ mixed thoroughly in 10:1:1 (v/v/v) ratio. 6 µL sample or standard, 18 µL distilled water and 180 µL FRAP working solution added respectively and incubated in dark condition at 37 ºC for 30 min. Absorbance was measured at 593 nm by using SpectraMax M3 and results were expressed as mM $FeSO_4$/kg dry sample.

15. Oxygen radical absorbance capacity assay

ORAC assay was carried out with few modification[51] to measure the antioxidant capacity of sorghum sample. Ten miligram of sample prepared in 1 mL of DMSO and diluted up to 30 µg per mL in 75 mM phosphate buffer at pH 7.4. Twenty five nanomole fluorescein and 25 mM AAPH also dissolved in phosphate buffer, consecutively. The total 200 µl of reaction mixture was prepare by adding 10 µL of sample, 90 of 25 nM fluorescein and 200 µl of AAPH. At 37 ºC for 120 min the fluorescein was measured every 3 min where excitation wavelength at 485 nm and an emission wavelength at 528 nm by SpectraMax M3. Antioxidant capacity was equivalent to mM TE/kg dry sample. The ORAC value was calculated by the following equation;

$$\text{ORAC value} = \text{Trolox Concentration}\left[\frac{(\text{AUC of sample} - \text{AUC of control}) \times \text{Dilution factor}}{\text{AUC of trolox} - \text{AUC of control}}\right]$$

16. α-glucosidase inhibitory activity

Alpha glucosidase (Sigma-Aldrich, USA) enzyme inhibition activity of *M. pupureus* K/M was carried out using 1 mM of PNPG as substrate according to Shi (2017)[52] with minor modifications. In 96 well plate, 10 µL of enzyme solution (0.23 U/ml), 50 µL of sodium phosphate buffer (50 mM, pH 6.8), 20 µl of distilled water and 10 µL of tested inhibitors in 1% DMSO were mixed and incubated at 37 °C for 10 min. After preincubation, 10 µl of substrate were added and at 37 °C for 10 min and the absorbance was recorded once per 2 min at 405 nm using by SpectraMax M3 (Molecular devices Inc., USA). One percent DMSO is used as a negative control. All tested inhibitors were analyzed in triplicate. The inhibitory percentage was calculated by the given formula:

$$\%\ \text{of inhibition} = \frac{\text{OD of control} - \text{OD of sample}}{\text{OD of control}} \times 100$$

17. Mushroom tyrosinase inhibitory activity

The mushroom tyrosinase inhibition of *M. purpureus* K/M fermented sorghum was determined as in our previous study[53]. Ten microliter of tyrosinase enzyme (20 U/mL), 10 µL of 50 mM sodium phosphate buffer pH 6.8, and 50 µL of sample were mixed together and incubate at 28 ºC for 10 min. After preincubation, 30 µL of L-dopa (3.3mM) added in the reaction at room temperature for 30 min and absorbance was measured once per 5 min at 475 nm using by SpectraMax M3. The final concentration of sample were between 0–7 mg/ml. The percent inhibition was calculated by the following equation:

$$\%\ \text{of inhibition} = \frac{\text{OD of control} - \text{OD of sample}}{\text{OD of control}} \times 100$$

18. Cell viability test

Macrophage RAW264.7 mouse cell line was purchased from Korean Cell Line Bank (Seoul, Korea). The macrophage cells were incubated at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gendepot, Massachusetts, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gendepot, Massachusetts, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Massachusetts, USA)[54-55]. RAW264.7 cell was seeded on 96 wells plate at 2×10^4 cells/well and cultured for 48 h. Before treated with non-fermented sorghum or fermented sorghum extract ranging from 125 to 1000 μg/mL in DMEM medium without Fetal bovine serum (FBS), cells were washed with phosphate-buffered saline (PBS). RAW 246.7 cells cultured in a medium without adding samples were used as controls. After 24 h, 10 μL of Ez-CyTox solution (Daeil Lab Service, Seoul, Korea) was mixed with 90 μL of medium and then incubated at 37 °C for 60 mins. Absorbance was measured at 450 nm using SpectraMax M3 and percent of cell viability was calculated.

Nitric oxide inhibitory activity

NO inhibitory activity was measured as previously described method [56]. RAW 2×10^4 264.7 cells/well were seeded to 96 wells plate and cultured at 37 °C. After 48 h 1 μg lipopolysaccharide (LPS)/mL added and then treated with sorghum or fermented sorghum extract in DMSO ranging from 62.5 to 250 μg/mL and cultured at 37 °C for 24 h. 80 μL of culture supernatant was mixed with 80 μL of Griess reagent for 20 min, and absorbance was measured at 540 nm using SpectraMax M3. The percent inhibition was calculated by the following equation:

$$\%\ \text{of inhibition} = \frac{\text{OD of control} - \text{OD of sample}}{\text{OD of control}} \times 100$$

Statistical Analysis

Experiments were conducted in triplicate, and the data were shown as mean ± standard error of the mean (SEM). Statistical analysis was done using one way ANOVA and Turkey *post hoc* multiple comparison tests on GraphPad Prism 8.0.2 (GraphPad Software, San Diego, CA, USA).

Results

1. Observation of sorghum surface

Surface of non-fermented sorghum and *M. purpureus* K/M fermented sorghum were observed by scanning electron microscopy at 50×, 100× and 200× magnification. Smooth mycelium and spore less surface was observed for non-fermented sorghum (Fig. 1a, c, e). *M. purpureus* K/M fermented sorghum was covered by spore (Fig. 1b, d). After removal of hyphae the degraded surface of *M. purpureus* K/M fermented sorghum grain was exposed (1f).

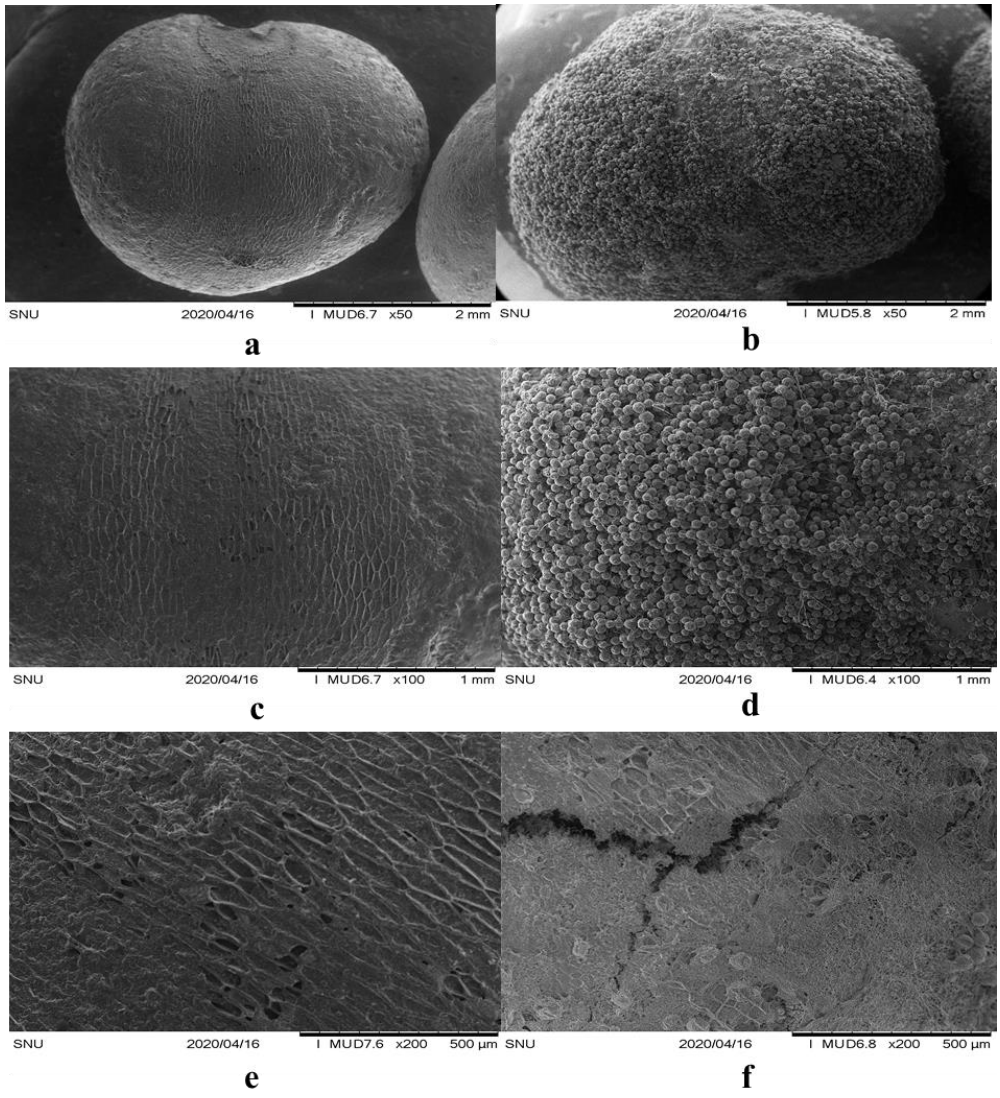

Fig 3 SEM analysis of fermented sorghum. a, non-fermented sorghum (50×); b, 7 day fermented sorghum (50×); c, non-fermented sorghum (100×); d, 7 day fermented sorghum; e, non-fermented sorghum (200×); f, 7 day *M. purpureus* K/M fermented sorghum (hypha removed) (200×).

2. Sorghum extraction by ethanol

Total 6 sorghum samples were extracted, among them 1 sample was non-fermented and other 5 samples were *M. purpureus* K/M fermented sorghum after 3, 5, 7, 10 and 14 day fermentation. The extraction yield keep increasing as fermentation day increased and highest extraction yield was achieved after 14 days fermentation, as shown in Table 1.

Table 1. Non-fermented and fermented sorghum extraction yields

	NF	3F	5F	7F	10F	14F
Extraction yields (%)	3.2	3.6	3.9	4.5	6.1	7

NF non-fermented sorghum, 3F 3 days, 5F 5 days, 7F 7 days, 10F 10 days, 14F 14 days fermented sorghum by *M. purpureus* K/M.

3. Analyses of L-carnitine

The amounts of L-carnitine in non-fermented sorghum was 0.42mg/kg (Table 2). In *M. purpureus* K/M fermented sorghum, fermented sorghum, amount of L-carnitine kept enhancing as the fermentation day increased. The highest L-carnitine amount was 120.6 mg/kg after 14 days fermentation by *M. purpureus* K/M. The total amount of L-carnitine synthesized by fungal fermentation increased 287 times compare to non-fermented sorghum (Table 2).

4. Analysis of phenolic compounds

Total 8 phenolic compounds (catechin, gallic acid, P-coumaric acid, caffeic acid, ferulic acid, vanillic acid, vanillin, protocatechuic acid) were analyzed in non-fermented and fermented sorghum by UPLC/MS system (Waters, Milford, MA, USA) (Table 2). The amount of catechin increased till 7 days fermentation as 8.9, 18.4, 64 and 65.1 mg/kg in NF, 3F, 5F and 7F, sequentially. After 10 days fermentation catechin amount decreased as 12.5 and 10.5 in 10F and 14F, respectively. Similar enhancement and reduction pattern as catechin was observed in caffeic acid during fermentation. Gallic acid and vanillic acid were appeared were not detected in non-fermented sorghum and appeared after fermentation. After 3 days fermentation protocatechuic acid, vanillin acid and ferulic acid concentration increased 14.3, 5 and 2.7 times compared to non-fermented sorghum. But protocatechuic acid and ferulic were keep decreasing whereas vanillin was stable throughout the fermentation. As for p-coumaric acid, 4.9 mg/kg was found in NF which keep decreasing as fermentation days increased (Table 2).

Table 2. L-carnitine and Phenolic compounds in non-fermented and fermented sorghum

Group (mg/kg)	NF	3F	5F	7F	10F	14F
L-carnitine	0.42±0.05*	30.2±0.1[a]	58.8±0.06[a]	64±0.2[a]	104.8±0.2[a]	120.6±0.7[a]
Catechin	8.9±0.8[*]	18.4±1.4[a]	64±0.8[a]	65.1±0.9[a]	12.5±0.3[c]	10.5±0.5[e]
Gallic acid	ND[*]	0.35±0.01[a]	0.41±0.02[a]	0.32±0.01[a]	0.34±0.04[a]	ND
P-coumaric acid	4.9±0.09[*]	2.8±0.02[a]	0.5±0.05[a]	0.3±0.04[a]	0.14±0.03[a]	ND
Caffeic acid	4.6±0.7[*]	14.7±1.3[a]	22.7±2.5[a]	15.2±0.7[a]	1.6±0.2[e]	1.8±0.1[e]
Ferulic acid	1.5±0.1[*]	4.1±0.3[a]	0.2±0.007[a]	0.32±0.08[a]	ND	ND
Vanillic acid	ND[*]	24.1±3.3[a]	47.8±4.6[a]	45.1±0.7[a]	35.6±0.6[a]	33.2±5.1[a]
Vanillin	0.1±0.03[*]	0.5±0.01[a]	0.5±0.03[a]	0.6±0.05[a]	0.6±0.07[a]	0.6±0.04[a]
Protocatechuic acid	2.1±0.2	30.3±1.9[a]	15.9±2[a]	12.5±0.1[a]	4.5±0.1[e]	3.9±0.4[e]

ND not detected, NF non-fermented sorghum, 3F 3 days, 5F 5 days, 7F 7 days, 10F 10 days, 14F 14 days fermented sorghum by *M. purpureus* K/M. All experiments were performed three times, and all data were showed as the mean ± standard deviation. [*] Control: Non-fermented, the different letters of alphabet represent significant differences ($p < 0.05$).

5. Total functional compound contents

Total phenolic, flavonoid and tannin contents of non-fermented and fermented sorghum are shown in Table 3. Compared to non-fermented sorghum total phenolic and flavonoid contents in *M. purpureus* K/M fermented sorghum increased from 807.8±6.8 to 1114.5±12.7 mg GAE/kg and 61.2±2.9 to 85.2±6 QE/kg after 5 day fermentation. After 10 day fermentation, phenolic and flavonoid contents decreased abruptly. As for total tannin contents, it keep decreasing as fermentation goes by. The tannin amount was decreased 2.9 times at 14 day fermentation in *M. purpureus* K/M fermented sorghum compare to non-fermented sorghum, respectively (Table 3).

6. Antioxidant activity of non-fermented and fermented sorghum

To evaluate the effect of fermentation in antioxidant activity ferric reducing antioxidant powder (FRAP), ORAC and DPPH scavenging activity were done. Ferric reducing power was increased from 12.7±0.5 mM Fe^{2+}/kg in non-fermented sorghum to 18.4±0.2 mM Fe^{2+}/kg in 5 day *Monascus ruber* fermented sorghum, sequentially (Table 3). In case of ORAC assay, antioxidant activity keep increasing till 5 days fermentation in *Monascus ruber* fermented sorghum from 80.8±3.2 TE mM/ kg to 119.1±2.3 TE mM/kg, respectively (Table 3). Like FRAP and ORAC assay, the scavenging activity of DPPH increased in fungal fermented sorghum after 5 day fermentation. In compare to non-fermented sorghum scavenging activity of *M. purpureus* K/M fermented sorghum 2 time increased. As shown in Table 3, a rapid fall in antioxidant activity was observed after 10 days fermentation in *M. purpureus* K/M fermented sorghum.

Table 3. Determination of total functional compound and antioxidant content in non-fermented and fermented sorghum

Group	NF	3F	5F	7F	10F	14F
TPC GAE[1] mg/kg	807.8±6.8*	890.9±2.9^{e}	1114.5±12.7^{a}	1082.7±4.5^{a}	670±75.2^{c}	513.4±5.3^{a}
TFC QE[2] mg/kg	61.2±2.9*	66.8±5.2^{e}	85.2±6^{b}	81.4±5.2^{b}	26.1±2.9^{a}	18.9±0.3^{a}
TCC TA[3] mg /kg	328.2±3.1*	276.1±4.5^{c}	215.9±2.2^{a}	186.3±5.6^{a}	155.5±5.8^{a}	113.5±4.6^{a}
FRAP Fe(II)[4] mM / kg	12.7±0.5*	13.8±0.04^{d}	18.4±0.2^{a}	17.7±0.4^{a}	11±0.4^{b}	8.1±0.3^{a}
ORAC TE[5] mM/ kg	80.7±3.2*	85.5±4.8^{e}	119.1±2.3^{a}	102.7±8.5^{c}	61.4±7.7^{d}	50.1±4.4^{b}
DPPH SC_{50}[6] mg/ mL	10.7±0.5*	7.5±0.3^{a}	5±0.2^{a}	5.9±0.08^{a}	13.8±0.2^{a}	16.4±0.04^{a}

NF non-fermented sorghum, 3F 3 days, 5F 5 days, 7F 7 days, 10F 10 days, 14F 14 days fermented sorghum by Monascus ruber. [1] gallic acid equivalent, [2] quercetin equivalent [3] tannic acid equivalent, [4] $FeSO_4$ equivalent, [5] trolox equivalent [6] concentration of 50% scavenging activity. All experiments were performed three times, and all data were showed as the mean ± standard deviation.

*Control: Non-fermented, each alphabet represent significant differences ($p < 0.05$).

7. Analysis of citrinin

Citrinin was analysed for sorghum fermented by *M. purpureus* K/M. Citrinin was not detected in any of the *M. purpureus* K/M fermented sorghum sample.

8. Detection of volatile compounds

In non-fermented and fermented sorghum, total 22 volatile compounds were detected. Out of 13 compounds, 2 acids, 1 terpene, 3 benzene derivatives, 2 alcohols, 1 ketones, 1 carbobicyclic, 1 alkyl aldehyde, 1 steroid and 1 ester were identified (Table 4). No significant change observed in total volatile content (TVC) after fermentation.

Table 4. Identification of volatile compounds (%)

No	Compounds	RT	Volatile compounds (%)			Aroma description
			NF	5F	7F	
	Acids					
1	Pentanoic acid	25.891	0.7%	ND	0.8%	Cheesy, sour milky, tobacco, fruity
2	Propanoic acid	25.89	ND	0.9%	ND	Pungent, acidic, cheesy, vinegar
	Terpene					
3	l-Limonene	11.797	1.3%	2.8%	2.2%	Citrus fruit
	Benzene Derivatives					
4	Styrene	7.882	ND	1.6%	ND	Sweet
5	Xylene	7.265	ND	ND	1.6%	Sweet
6	Benzene	7.257	1.5%	1.8%	ND	Sweet, gasoline
	Ketone					
7	2-Propanone	2.396	91.1%	87.9%	90.6%	Sweet, caramel, fruity, spicy radish, acetone like
	Alcohol					
8	1-Pentanol	4.277	1.5%	ND	ND	Apricot, Pungent, fermented, bread, yeast, fusel, wine
9	(1-D)ethenol	5.534	ND	ND	1.9%	Alcohol
	Alkyl aldehyde					
10	Hexanal (CAS)	5.532	ND	2.7%	ND	Fruity, grass, leafy, sweaty
	Ester					
11	Ethyl Acetate	2.922	ND	3.2%	3.1%	Sweet, glues, nail polish remover
	Amine					
12	1,4-Butanediamine	2.912	4.2%	ND	ND	Fishy, ammoniacal, pungent
	Steroid					
13	1-Monolinoleoylglycerol trimethyls	30.58	11.4%	ND	ND	

RT retention time, ND not detected, NF non-fermented sorghum, 5F 5 days, 7F 7 days fermented sorghum by *M. purpureus* K/M.

9. Antidiabetic activities

The α-glucosidase inhibitory effects of fermented sorghum was investigated in this study. The inhibitory percent of α- glucosidase increased as the fermentation day increased till 5 days and suddenly dropped after 10 days fermentation. The IC_{50} value of non- fermented sorghum was 71.4±1.8 mg. IC_{50} values of 3-14 day *M. purpureus* K/M fermented sorghum were 58.2±0.5 mg, 35.7±2.7 mg, 39.3±2.1 mg, 96.5±2.1 mg and 101±4 mg (Fig 2), respectively.

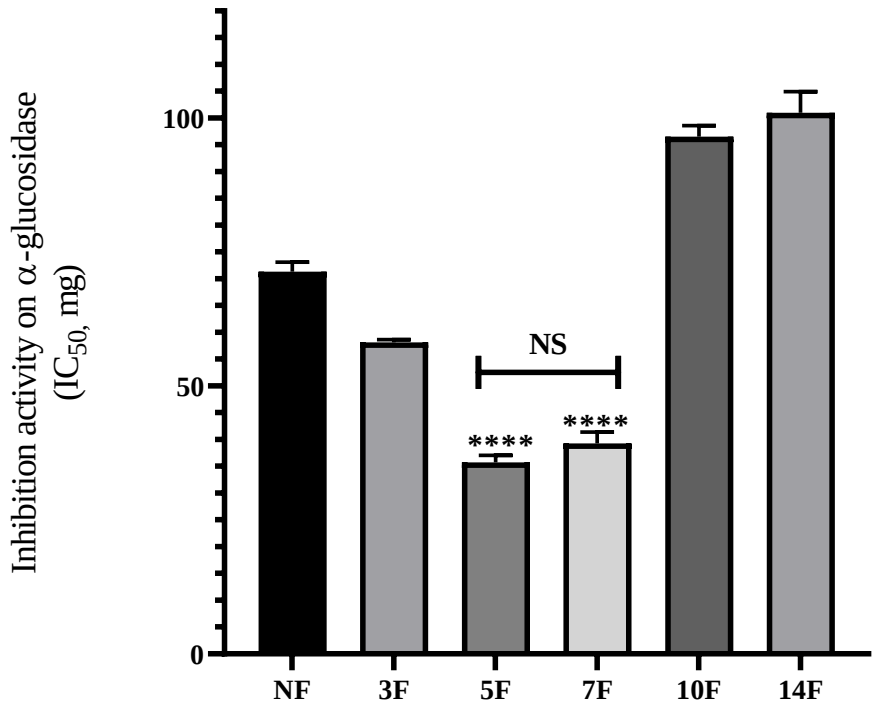

Fig 4 Inhibitory activity of non-fermented and fermented sorghum against α-glucosidase enzyme. NF non-fermented sorghum, 3F 3 days, 5F 5 days, 7F 7 days, 10F 10 days and 14F 14 days M. *purpureus* K/M fermented sorghum. All experiments were performed three times, and all data were showed as the mean ± standard deviation. ****p<0.0001 was considered statistical significance of 5F compared with the control (NF). NS no significance.

10. Mushroom tyrosinase inhibitory activity

In this experiment, mushroom tyrosinase inhibitory activity of non-fermented and *M. purpureus* K/M fermented sorghum were studied. The IC_{50} value of non- fermented sorghum was 7.4±0.1 mg. For *M. purpureus* K/M fermented sorghum of 3-14 day IC_{50} values were 6.3±0.09 mg, mg, 3.1±0.1 mg, 3.7±0.5 mg, 12.1±0.1 mg and 12.9±0.09 mg (**Fig 3**), respectively. Compare to non-fermented sorghum, 5 days fermented sorghum have 2.4 times higher inhibition.

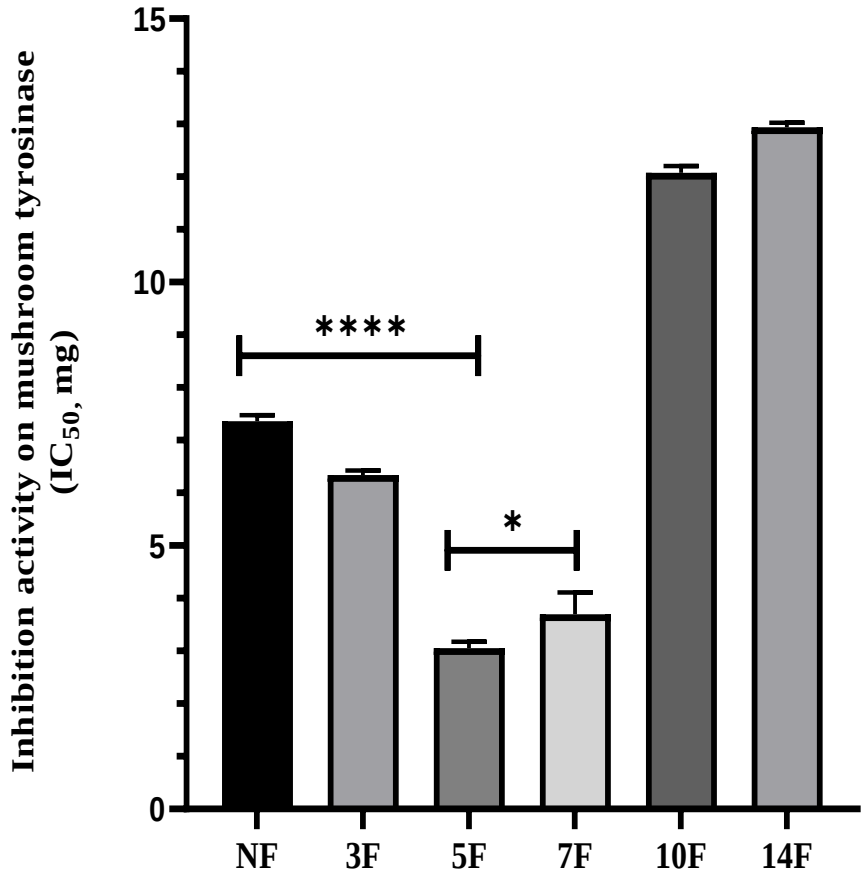

Fig 5 Inhibitory activity of non-fermented and fermented sorghum against mushroom tyrosinase enzyme. *NF* non-fermented sorghum, 3F 3 days, 5F 5 days, 7F 7 days, 10F 10 days and 14F 14 days *M. purpureus* K/M fermented sorghum. All experiments were performed three times, and all data were showed as the mean ± standard deviation. ****p<0.0001, *p<0.0153 was considered statistical significance of 5F compared with the control (NF) and 7F, respectively.

11. RAW264.7 cell viability

Cell viabilities of non-fermented and fermented sorghum treated RAW 264.7 macrophages cells was shown in Fig 4. Depending on the fermentation time and sample concentration cell viabilities changed. At 1000, 500, 250 and 125 μg/mL concentration cell viabilities were 53.2±3.1%, 110.5±1.2%, 106.4±0.09% and 108.4±1.4% for 3 days fermented sorghum, 51.9±0.8%, 103.6±0.1%, 104.9±0.5% and 105.9±0.3% for 10 days fermented sorghum, 66.3±1.1%, 111.3±0.6%, 109.7±4% and 105.6±0.2% for 10 days fermented sorghum, respectively. 3 and 7 days fermented sorghum showed 100% cell viability at 250 μg/mL concentration as non-fermented sorghum above 90% cell viability was observed at same concentration. At 250 μg/mL concentration cell viabilities reached 100% for all sample as a result the nitric oxide inhibition assay was carried out from 250, 125, and 62.5 μg/mL (Fig 4).

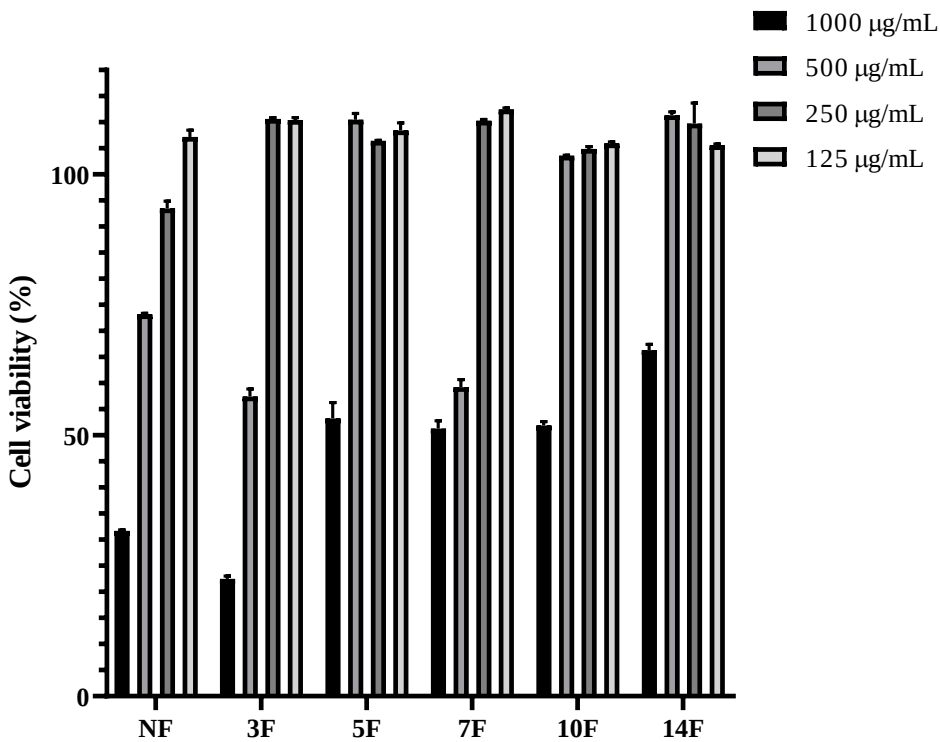

Fig 6 Cell viability on RAW264.7 cells of non-fermented and fermented sorghum. *NF* non-fermented sorghum, 3F 3 days, 5F 5 days, 7F 7 days, 10F 10 days and 14F 14 days *M. purpureus* K/M fermented sorghum. non-fermented and fermented sorghum. All experiments were performed three times, and all data were showed as the mean ± standard deviation.

12. Nitric oxide inhibition assay

Nitric oxide inhibitory assay was investigated by inoculation of lipopolysaccharides (LPS) in culture media which stimulate the production of nitric oxide. As the amount of non-fermented and fermented sorghum increased the nitric oxide inhibitory activity also enhanced. No significate change in nitric oxide production was observed between non-fermented and fermented sorghum at the concentration of 250 μg/mL. As Nitric oxide production caused by inflammation, fermented sorghum can use as anti-inflammatory agent (Fig 5).

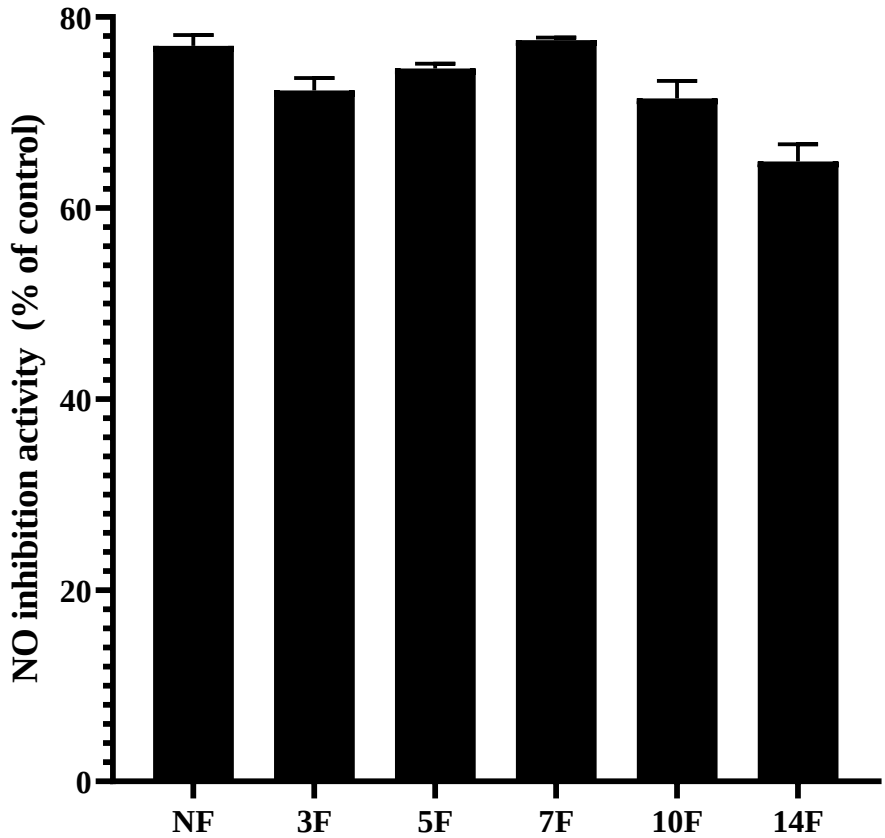

Fig 7 NO Inhibitory activity of non-fermented and fermented sorghum. *NF* non-fermented sorghum, 3F 3 days, 5F 5 days, 7F 7 days, 10F 10 days and 14F 14 days *M. purpureus* K/M fermented sorghum. All experiments were performed three times, and all data were showed as the mean ± standard deviation.

Discussion

Sorghum is super crop as well as one of the fifth highest cultivating crop in the world. Sorghum have been used as a raw material to make bread, porridges, grain tea and beer in many region of Asia and Africa[57-58]. Although sorghum is rich in health beneficial bioactive compounds compare to any other cereal but it mainly used for animal feed[59]. Recently, consumption of sorghum as food is increasing day by day due to its unique phenolic compounds profile, which related with the reduction of chronic diseases like type-2 diabetes, cardiovascular diseases, and cancer risk. The aim of this study is to development of functional food using sorghum by improving the nutritional and biochemical properties using solid-state fermentation.

After 7 day fermentation using *M. purpureus* K/M, degradation of sorghum surface was observed by SEM (Fig 3). During fermentation like *R. oligosporus, Monascus* spp. secrete hydrolytic enzymes like lipases, amylases, protease, and glucoamylase which responsible for the surface degradation as well as complex cell well bound compounds into simpler substances[23, 60-61].

Our previous study showed that highest l-carnitine obtained from *R. oligosporus* fermented quinoa was 3.15 ± 0.06 mg/kg after 3 day fermentation and decreased to 1.54 ± 0.06 mg/kg after 5 day fermentation[24]. In case of our current study, *M. purpureus* K/M fermented sorghum L-carnitine amount keep rising as fermentation day increased. 120.6±0.7 mg/ kg (Table 2) was the highest amount of L-carnitine amount after 14 day fermentation by *M.*

purpureus K/M (Table 2). Although limiting amino like lysine (substrate for L-carnitine synthesis) is low in sorghum grain, it did not affect the production of L-carnitine in sorghum. As studies showed that during fermentation the amino acid content of grain increases[62-63] reported that, after fermentation with *R. oligosporus* lysine increased from 141.5 mg/100 g to 176.8 mg/100 g. Another study showed that after fermentation lysine and methionine amount in sorghum increased from 8.75±0.03 to mg/100g to 14.35±0.02 to mg/100g and 1.11±0.01 to mg/100g to 3.87±0.01 to mg/100g, respectively[64].

Citrinin was analyses by UPLC/MS system (Waters, Milford, MA, USA) with QDa detector. Citrinin is one of the secondary metabolite besides pigments and monacolin-k produce by *Monascus spp.* during fermentation[65-66]. Citrinin is a mycotoxin that have nephrotoxicity and hepatotoxicity, which can effect both human and animal. That is why many countries limited the use of *Monascus* fermented produces. 50 μg/kg citrinin in food is the maximum acceptable level in Korea[67]. No citrinin was detected in *M. purpureus* K/M fermented sorghum till 14 day fermentation.

Phenolic acids, flavonoids and condensed tannin are the major phenolic compounds presents in sorghum[68]. Although all sorghum contain phenolic acids in bound or unbound form but not all sorghum contain condense tannin as well as anthocyanins which is only present in pigmented sorghum[68]. Phenolic acids like protocatechuic acid, caffeic acid and vanillin concentration increased 7.6, 5 and 5 times after 5 days fermentation, sequentially. Hydroxybenzoic acids like gallic acid and vanillic acid, which

were not detected in non-fermented sorghum, emerged during fermentation. While on the contrary ferulic acid and p-coumaric acid amount reduced during fermentation. Metabolic pathways like glycosylation, deglycosylation, ring cleavage, methylation, and glucuronidation are responsible for bioconversion of phenolic compound in the course of fermentation[69]. Which explain the increasing, decreasing and formation of new phenolic compounds thought out fermentation. TPC of fermented sorghum increased 807.8±6.8 to 1114.5±12.7 mg/kg as fermentation day increase and after 10 day fermentation TPC decreased (Table 3). Fungal solid state fermentation of cereal improve the phenolic content by secreting various hydrolyzing enzymes like α-amylase, β-glucosidase, xylanase and cellulase which are produce by fungus during fermentation[70-73]. Some study reported that *Monascus* spp. could produce cellulose, xylanase, esterase, α-amylase, β-glucosidase and β-glucuronidase fermentation[74-76]. These enzymes can degrade the surface of grain by break down the cell well and realize the cell well bound phenolic compound by hydrolysis of glycosidic bonds. During sorghum fermentation with *M. purpureus* K/M phenolic acids like caffeic acid, gallic acid, vanillic acid and protocatechuic acid concentration increased as a result total phenolic content also improved (Table 2)[75] Bei (2018) also reported the enhancement of phenolic acid in oats during fermentation with *Monascus anka*. Catechin one of the flavonoids and secondary metabolites of cereal increased 8.9±0.8 to 64±0.8 mg/kg after fermentation which explained enhancement of TFC of fermented sorghum (Table 2 and 3). Increasing of

both TPC and TFC during fermentation was also observed in our previous study[24].

Hydrogen atom transfer (HAT) and single electron transfer (SET) are the main antioxidant activity present in plant[77]. Hence, DPPH, FRAP and ORAC assay were done to check the antioxidant capacity of fermented sorghum as ORAC is a hydrogen atom transfer antioxidant assay which is similar to the in vivo antioxidant system[78]. Whereas, FRAP and DPPH both electron transfer antioxidant assay but FRAP is more sensitive to secondary metabolites and peptides produce during fermentation compared to DPPH which is mainly used to detect hydrophobic antioxidants[79]. Fermented sorghum antioxidant activity increased 1.5 times including FRAP and ORAC. In case of DPPH, antioxidant activity increased 2.1 times compare to control. Highest antioxidant activity was recorded at 5 days fermentation and rapidly decreased after 10 days fermentation. Cheng (2016) reported improvement of antioxidant activity of rice bran after fermentation by *Monascus pilosus* KCCM60084[79]. Moreover, after fermentation increasing of antioxidant was reported by Mohaparta (2019) and Zaroug (2014)[64, 80]. Antioxidant activity of sorghum and total phenolic content are strongly correlated[81]. So enhancement of antioxidative activity of fermented sorghum was related to the TPC and TFC amount during fermentation[82].

Significance reduction of tannin content observed compared to non-fermented sorghum. Throughout, fermentation total tannin content dropped from 328.2±3.1 mg/kg to 113.5±4.6 mg/kg in sorghum. One study showed[83]

notable reduction of tannin content in sorghum during fermentation, another study[46] reported reduction of tannin in fermented coffee.

Phenolic compounds like caffeic acid, protocatechuic acid, vanillic acid and gallic acid amount increase after fermentation with *M. purpureus* K/M which are present in sorghum and *Monascus* fermented products and can play important roles in inhibition of α-glucosidase[84-85]. May be that's why, after 5 days fermentation the inhibitory activity of α-glucosidase increased, remarkably. A positive correlation found between the phenolic compounds in sorghum and α-glucosidase inhibition[35] and several studies reported elevation of phenolic contents in *Monascus* fermented products in contrast to non-fermented[86-87].

Antityrosinase activity of *M. purpureus* K/M fermented sorghum improved 241.4% compare to non-fermented sorghum. Antioxidant also improved during fermentation in sorghum and a positive correlation was found between tyrosinase inhibition and antioxidant activity[88]. Castro-J´acome (2021) reported that peptides of kafirins the main protein of sorghum have tyrosinase inhibitory activity[39]. *M. purpureus* K/M can hydrolyzed kafirins which can be one of possible reason for the improvement of tyrosinase inhibition during fermentation.

There was no significant inhibitory difference observed between non-fermented and fermented sorghum against NO production in LPS-induced RAW264.7. Similar result observed in Lee (2020) where no significant difference in NO inhibition showed between non-fermented and fermented

ginseng[89]. One of the study reported that, the flavonoids block the expression of iNOS enzyme in lipopolysaccharide-activated RAW264.7 cells and reduce NO production[90]. Burdette reported that extract of black sorghum block TNF-α and IL-1β release in LPS-stimulated blood mononuclear cells[91].

Conclusion

In conclusion, our studies reported about production of l-carnitine by fermentation using *M. purpureus* K/M in sorghum for the first time. Citrinin was not detected in *M. purpureus* K/M fermented sorghum. Phenolic compound like vanillic acid and gallic acid which were not present before fermentation appeared as well as catechin, caffeic acid and protocatechuic acid amount also increased significantly. Total phenolic and flavonoid compounds increased which enhance the antioxidant activity of fermented sorghum compare to non-fermented one. Anti-diabetic as well as tyrosinase inhibition activity increased in significate amount and 74.6% NO inhibition was observed in 5 days fermented sorghum. During fermentation tannin, one of the anti-nutrient compound in sorghum decreased which increase the digestibility of sorghum and no significant changes occur in aromatic compounds in fermented sorghum. Based on these finding, we suggest fermented sorghum can be used for development of functional food.

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Abstract in Korean

수수 (*Sorghum bicolor*)는 슈퍼 작물 중 하나이며, 다섯 번째로 영양가가 높은 주요 곡물이다. 수수는 페놀산, 플라보노이드 및 축합 탄닌이 주성분이며, 다양한 페놀 화합물 함량이 높은 글루텐 프리 곡물이다. 본 연구의 목적은 발효를 통해 L-carnitine과 수수의 다른 기능성 성분을 증가시키기 위함이다. 발효 샘플을 이용하여 SEM과 같은 형태학적 연구, LC/MS (L-carnitine, citrinin and phenolic compounds)와 같은 분석 연구, TPC, TFC, TTC, DPPH, FRAP 및 ORAC와 같은 생화학적 연구를 수행하였다. 미발효 수수의 매끈한 표면이 *Monascus purpureus* K/M에 의한 발효 과정 이후 수수 외부 표면의 분해가 관찰되었다. L-carnitine 양은 *M. purpureus* K/M에 의한 14일 발효 후 0.42±0.05 mg/kg에서 120.6±0.7 mg/kg으로 증가하였다. 총 페놀 및 총 플라보노이드 함량은 발효 과정 동안 증가하였다. 반면, 총 탄닌 함량은 발효일이 증가함에 따라 290%로 감소하였다. DPPH, FRAP 및 ORAC 분석에서는 발효되지 않은 수수에 비해 항산화 활성이 증가하는 것으로 나타났다. 발효 후 갈산과 바닐산이 생성되었다. α-glucosidase와 버섯 tyrosinase의 저해 활성도 발효 5일 후 199.6%, 141.4% 증가하였다. *M. purpureus* K/M에 의한 발효 후 수수의 L-carnitine 함량은 287배 증가하였다. 발효수수의 세포독성은 비발효수수에 비해 감소하였다. 대식세포에 대한 NO 생성 억제율은 각각 76.9%, 74.6% 및 77.6%로 각각 비발효, 5일 및 7일 발효 수수에서 관찰되었다. 따라서 본 연구를 바탕으로, 발효한 수수는 식품, 제약, 화장품 산업에서 기능성 소재로 응용 가능할 것이라 기대된다.

Keywords: Sorghum, Fermentation, L-carnitine, Anti-inflammatory activity, *Monascus purpureus*.

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