Tropical Journal of Pharmaceutical Research January 2023; 22 (1): 89-97
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v22i1.13

Original Research Article

Marsilea crenata extract modulates SOD1 expression in male rat testes via Nrf2 pathway

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Sent for review: 15 August 2022 *Revised accepted: 18 December 2022*

Abstract

Purpose: *To investigate the potential effect of M. crenata ethanol extract as source of antioxidants through SOD1 and Nrf2 expression levels in rat testes.*
Methods: *Phytochemical analysis of M. crenata was performed using high-performance liquid chromatography (HPLC), while antioxidant activity was determined by 2.2-diphenyl-1-picrylhydrazyl (DPPH radical) assay. This study used 24 male rats (n = 6), divided into 4 groups: control (normal rat without any treatment); PS1, PS2, and PS3 (normal rat which orally received ethanol extract of M. crenata leaves at a dose of 43.2, 86.4, and 129.6 mg/200 g body weight for 30 days, respectively). The expression levels of Nrf2 and SOD1 were analyzed by flow cytometry.*
Results: *Quercetin and genistein were found in ethanol extract of M. crenata leaves with retention times of 8.812 and 22.309 min, respectively. M. crenata leaves extract was categorized as a strong antioxidant (IC_{50}: 33.68 ppm). There was an increase in Nrf2 expression in rat testis in PS1 group. An upregulation of SOD1 expression was found in PS2 group.*
Conclusion: *M. crenata extract is a rich natural source of antioxidants and phytoestrogen and thus may be useful in the management of testicular dysfunctions. More studies will be required to firmly establish this finding.*

Keywords: *Antioxidant, Marsilea crenata, Genistein, Nrf2, SOD1, Phytoestrogen, Quercetin*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Mammalian testes are dynamic organs with two main functions producing spermatozoa through spermatogenesis and testosterone through steroidogenesis. Spermatogenesis is a complex process that occurs in the seminiferous tubules of the testes and includes varied cellular activities such as proliferation, meiosis, and differentiation [1]. Likewise, steroidogenesis in Leydig cells is a multi-step process that requires several enzymes to convert cholesterol to testosterone. Spermatogenesis and steroidogenesis are two physiological processes that can produce reactive oxygen species (ROS) [2]. Recent studies demonstrated that ROS plays a role in testicular function. ROS plays a role in germ cell apoptosis, capacitation, hyperactivation, and acrosome reactions of spermatozoa [3]. However, excessive ROS

results in oxidative stress, thereby inducing lipid peroxidation, and damage to DNA, RNA, spermatozoa protein function, and other testicular cells. So, maintaining a balance of ROS levels is very important to maintain normal cell function. Antioxidants can play a role in maintaining ROS levels by counteracting or preventing their formation in testicular cells [4].

Based on their source, antioxidants are classified as exogenous and endogenous antioxidants. Exogenous antioxidants are antioxidants obtained from the diet, such as carotenoids, vitamin E, and some flavonoid compounds, such as quercetin, rutin, apigenin, naringin, and hyperosida [5]. Endogenous antioxidants are antioxidants that are synthesized in the body, such as GSH (Glutathione), SOD (Superoxide Dismutase), and CAT (Catalase). One of the molecules that play a role in synthesizing endogenous antioxidants is Nrf2 (nuclear factor erythroid-2-related factor 2), a transcription factor member. In an inactive state, Nrf2 binds to Keap1 in the cytoplasm. When the interaction between Keap1 and Nrf2 is inhibited, Nrf2 is activated, and Nrf2 moves toward the nucleus to regulate some endogenous antioxidants [6].

ROS can induce the dissociation of Keap 1-Nrf2 interactions from cell metabolism or diets containing flavonoids. Flavonoids can bind to amino acid residues of Keap1 protein, which causes changes in Keap1 conformation. The conformational change of Keap1 causes the dissociation of the Keap1-Nrf2 interaction. Some flavonoid groups have potential for Keap1 and Nrf2 dissociation, including curcumin [7], genistein, and rutin [8].

Indonesia has more than 38,000 species of plants, which are potentially used as traditional herbal medicine. Water clover (*Marsilea crenata*) is a species of wild plant that is consumed by the local communities and contains a lot of flavonoids. *M. crenata* has various flavonoid compounds, including naringenin, hyperosida, daidzein, and genistein [9]. To maintain normal testicular function, researchers have explored plants that can enhance antioxidant expression in the testes. This study aimed to determine the potential of water clover (*M. crenata*) ethanol extract as source of antioxidants through the expression level of Nrf2 and SOD1 in rat testes.

METHODS

Extract preparation

Marsilea crenata extract was prepared using maceration method. Powdered *M. crenata* (350 g) were extracted with 3 L of 70 % (v/v) ethanolic solution at room temperature for 24 h. The extract was evaporated using a rotary evaporator at 80 rpm, and 4 ^{o}C. The extract was then dehydrated using a water bath at 70 ^{o}C. The sample was then stored at −20 °C until further use.

Quercetin and genistein analysis

High-performance liquid chromatography (HPLC) apparatus system (LC-Prominence-20 AT and SPD 20A UV-Vis detector, Shimadzu Co., Tokyo, Japan) was used to identify the quercetin and genistein compounds in the ethanol extract of *M. crenata* by comparing it with standard quercetin and genistein. The HPLC analysis was performed according to Lone *et al* [10]. Separations were done by Shim-pack VP ODS C18 (150 x 4.6 mm, 5 µm, Shimadzu Co., Tokyo, Japan) reverse-phase column. Five microliter samples were injected with a column temperature (CTO 10 ASVP) of 25 °C. The wavelength detector was 280 nm. The flow rate was 1.0 mL/min at the following mobile phase composition: 0.1 % phosphoric acid in water (A), acetonitrile (B) 75/25 v/v, using an isocratic method total run time of 40 min. Each compound identification was made by comparing the retention time and ultraviolet absorption spectrum with several standards.

2.2-Diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity

The DPPH free radical scavenging activity of ethanol extract of *M. crenata* was carried out spectrophotometrically with different concentrations of *M. crenata* (0, 5, 10, 15, 20, 25, 40, 50, and 60 ppm). The extract was added to an equal volume, 1 mL samples, and 1 mL DPPH solution. Samples were carried out in triplicate and incubated for 30 min at 37 ^{o}C. The absorbance was measured at 517 nm with ultraviolet and visible spectrophotometry. Vitamin C was used as a standard reference. The percentage of inhibition (I) was calculated using Eq 1.

I (%) = {(CA-SA)/CA}100 …… (1)

where CA = control absorbance, and SA = samples absorbance

Animals

Twenty-four adult male rats (4 months old and weighing 200 - 300 g) were obtained from Biosains Institute, Brawijaya University, Malang. Rats were kept in plastic cages and supplied with

a standard pellet diet and tap water under a 12 h light/dark cycle and room temperature of about 25 °C. This study was approved by the Ethics Committee of Brawijaya University (approval no. 1067-KEP-UB) and followed the guidelines of the Declaration of Helsinki [11].

Protocol

A total of 24 male rats were divided into 4 groups (n = 6). The groups were treated as follows: control (normal male rat without any treatment); P1, P2, and P3 (male rats orally received ethanol extract of *M. crenata* leaves at doses of 43.2, 86.4 and 129.6 mg/200 g, for 30 days, respectively. On the 31st day, the rats were sacrificed and dissected, and the testes were isolated.

Determination of Nrf2 and SOD1 expressions

Cell isolation

The right testis of the rat was dispersed using syringe in 5 mL Phosphate Buffered Saline (PBS). Homogenate was filtered using wire and added into a 15 mL propylene tube. Then, the homogenate was centrifuged at 2500 rpm, for 5 min at 10 °C. The supernatant was removed, and the pellet was resuspended with 1 mL PBS. Fifty microliter of cell suspension was added with 0.5 mL PBS and then centrifuged at 2500 rpm, for 5 min, 10 °C. The supernatant was removed, and the pellet was used for the next antibody staining protocol.

Immunostaining and flow cytometry analysis

Pellet was added with 50 µL of cytofix-cytosperm and incubated for 20 min at 4 °C in the darkroom. Then, it was added with 500 µL intracellular staining permeabilization wash buffer. Homogenate was centrifuged at 2500 rpm, for 5 min at 10 °C. Pellet was added with 50 µL primary antibody against Nrf2 and SOD1, then incubated for 20 min at 4 °C. Then, the samples were added with 50 µL secondary antibody and incubated for 20 min, at 4 °C. The samples were then added with 400 µL PBS and transferred into a flow cytometry cuvet. The samples were then run into a flow cytometer (BD Biosciences FACS Calibur™).

In silico analysis

In silico analysis was used to support SOD1 and nrf2 expression data by analyzing the interaction between the protein target (Keap1 and SOD1) and the bioactive compound of *M. crenata*. Two compounds from HPLC analysis were selected. The three-dimensional structure of genistein (CID 5280961) and quercetin (CID 5280343) were taken out from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The antioxidant protein, including superoxide dismutase-1 (SOD1), Kelch-like ECH-associated protein 1 (Keap1), and Nrf2, were downloaded from the Protein data bank (PDB) (1Hl5, 5fzn, and 5wfv, respectively). Quercetin and genistein interacted among SOD1, Keap1, and Nrf2 by HEX Cuda 8.0 program and were analyzed by Discovery studio version 21.1.1.0 software. The data were shown in three- and two-dimensional views and tabulated.

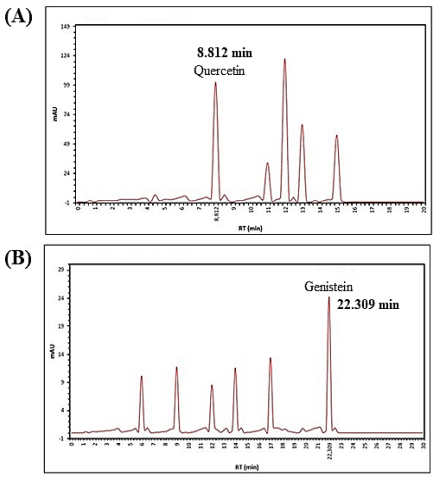
Statistical analysis

The data of Nrf2 and SOD1 expressions were analyzed statistically using SPSS 20.0 for Windows. Statistical significance between group treatments was performed by one-way analysis of variance (ANOVA) followed by the Tukey test ($p \leq 0.05$), while *in silico* and HPLC data were analyzed descriptively.

RESULTS

HPLC chromatogram

The HPLC analysis of the ethanol extract of *M. crenata* showed the presence of quercetin and genistein (Figure 1). It indicated that both compounds were present in *M. crenata* extract in significant quantities. Retention times examined for genistein and quercetin were 8.812 and 22.309 min.



(C)

Compound	Weight (g)	Retention time (min)	Sample curve area	Concentration (µg/g)
Quercetin	0.102	8.812	101.72	1116.16
Genistein	0.108	22.309	24.31	624.20

Figure 1: HPLC chromatogram of two standards in *Marsilea crenata* samples. (A) Quercetin and (B) Genistein, (C) The retention time of the two standards

Antioxidant activity

Ethanol extract of *M. crenata* was investigated for free radical scavenging activity with DPPH assay. Based on linear regression (Y = 5.961 x + 4.719, r = 0.981), the IC_{50} of *M. crenata* extract was 33.68 ppm, and vitamin C was 7.6 ppm (Figure 2). The IC_{50} of the ethanol extract of *M. crenata* was lower than 50 ppm and can therefore be categorized as a strong antioxidant.

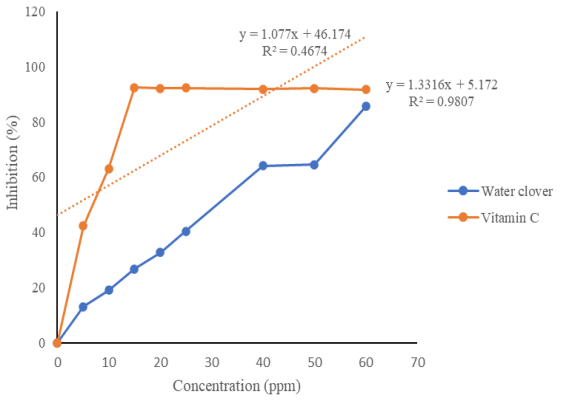


Figure 2: DPPH antioxidant activity of ethanol extract of *Marsilea crenata*

Expressions of Nrf2 and SOD1 in rat testes

In this study, alterations in Nrf2 and SOD1 levels in rat testes after treatment with *M. crenata* extract were validated by flow cytometry analysis. The FACS dot plot analysis demonstrated that 43.2 mg/200 g BW of *M. crenata* (PS1) extract could increase Nrf2 expression levels in the testis of rats compared to control rats ($p < 0.05$) (Figure 3 A). The PS2 and PS3 groups did not impact Nrf2 expression in testis, as indicated by reduced levels of Nrf2 in both groups (Figure 3 B).

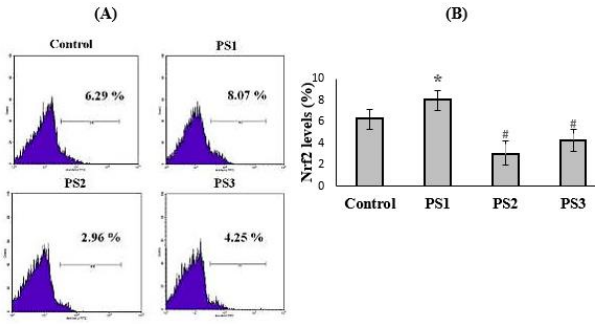


Figure 3: *M. crenata* extract increased Nrf2 levels in male rat testis. Control: without any treatment; PS1-PS3: *M. crenata* extract at doses of 43.2 mg/200 g BW, 86.4 mg/200 g BW and 129.6 mg/200 g BW, respectively. Values are expressed as mean ± SEM (N = 6), *$p < 0.05$ vs. control; #$p < 0.05$ vs PS1

The study also found that *M. crenata* extract at dose 86.4 mg/200 g BW (PS2) showed an upregulation of SOD-1 expression in male rat testis (Figure 4 A). PS1 and PS3 were not significantly different from the control group ($p > 0.05$) (Figure 4 B). It was indicated that PS1 and PS2 could not lead to activation of SOD1 in the testicular organ.

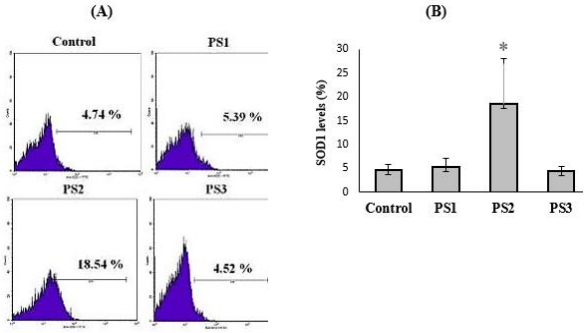


Figure 4: *M. crenata* extract increased SOD1 levels in male rat testis. Control: without any treatment; PS1-PS3: *M. crenata* extract at doses of 43.2 mg/200 g BW, 86.4 mg/200 g BW, and 129.6 mg/200 g BW, respectively. Values expressed as mean ± SEM (N = 6), * P 0.05 vs. other treatment

Interaction between bioactive compound of *M. crenata* and SOD1 protein

Quercetin binds to the SOD1 protein in seven amino acid residues, which is higher than genistein (Figure 5, Table 1). The LYS9 residue was found in both quercetin-SOD1 and genistein-SOD1 interactions (Figure 5 a and b). Quercetin and genistein interacted with superoxide dismutase (SOD1) protein through hydrogen bonds, hydrophobic interactions, and unfavorable bumps. Various types of interactions affected the quercetin-SOD1 complex, which has lower binding energy than genistein-SOD1.

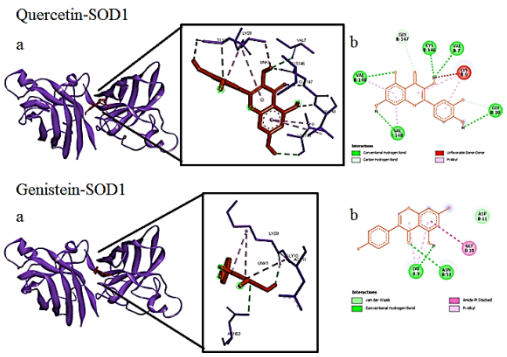


Figure 5: The binding poses among quercetin, genistein, and SOD1. (A) Quercetin and genistein showed in a red color, and SOD1 protein was illustrated in a purple ribbon structure; (B) 2D structure of the ligand-protein complex

Table 1: Interaction among quercetin, genistein and SOD1

Ligand-Protein complex	Binding energy (kJ/mol)	Residues (distance in A)	Category	Types of interaction
Quercetin-SOD1	-182.5	**B:VAL148:N(**3.18); B:GLY10:O (2.71); B:VAL7:O2.54 B:CYS146:O2.18 I:VAL148:O2.83 **B:GLY10:CA**3.25 **B:GLY147:CA(**3.18)	Hydrogen Bond	Conventional Hydrogen Bond
		B:LYS95.45 B:VAL1484.94 I:VAL148(5.29)	Hydrophobic	Pi-Alkyl
		B:LYS9(4.02)	Hydrophobic	Pi-Alkyl
		B:LYS9:N2.69	Unfavourable	Unfavourable Donor-Donor
Genistein-SOD1	-170	**B:ASN53:ND2(**3.23); B:LYS9:O2.18	Hydrogen Bond	Conventional Hydrogen Bond
		B:GLY10:C.O;ASP11:N(4.48)	Hydrophobic	Amide-Pi Stacked
		B:LYS9(4.35) B:LYS95.40	Hydrophobic	Pi-Alkyl

The bioactive compound of *M. crenata* as activator Keap1/Nrf2

Based on *in silico* analysis, the Nrf2 protein interacted with the Keap1 protein in several residues (Figure 6, Table 2). Quercetin and genistein were bound to Nrf2 and Keap1 proteins in several residues, with some of them being found on the Nrf2–Keap1 complex protein (Figure 6). Quercetin bound to Keap1 in the same site of Nrf2, which were VAL324, ARG470, VAL467, VAL608, VAL514, ALA466, ALA607, ASN469, and THR560 (Table 3). Moreover, genistein determined in *M. crenata* extract when interacting with Keap1, the Nrf2 change the binding site region of Nrf2-Keap1 complex protein. These findings indicated that quercetin inhibited the Nrf2-Keap1 interaction by substituting the Nrf2 binding site as a native ligand.

DISCUSSION

The present study found that *M. crenata* extract had a high antioxidant activity which was categorized as an exogenous antioxidant. Exogenous antioxidants, such as phenolic, could enhance Nrf2 activation and cause it to release its bonds with Keap1. Therefore, the transcription of antioxidant genes occurs through Nrf2/ARE pathway. Furthermore, antioxidants reduce oxidative stress indirectly by inducing phase II detoxification enzymes and antioxidant genes, which results in the production of endogenous antioxidants. Flavonoids with hydroxyl groups on the C ring, such as quercetin and myricetin, were the most effective inducers. It has been reported that genistein and quercetin have a protective effect on the reproductive organ [12]. Zhang *et al* [13] demonstrated that genistein partially attenuated DEHP-induced male reproductive disorder by activating Nrf2/HO-1 in prepubertal male Sprague-Dawley rats. Yuan *et al* [14] revealed that quercetin could protect against PFOA-induced testicular toxicity by increasing Nrf2 and its target antioxidant genes such as SOD-1, CAT, and HO-1.

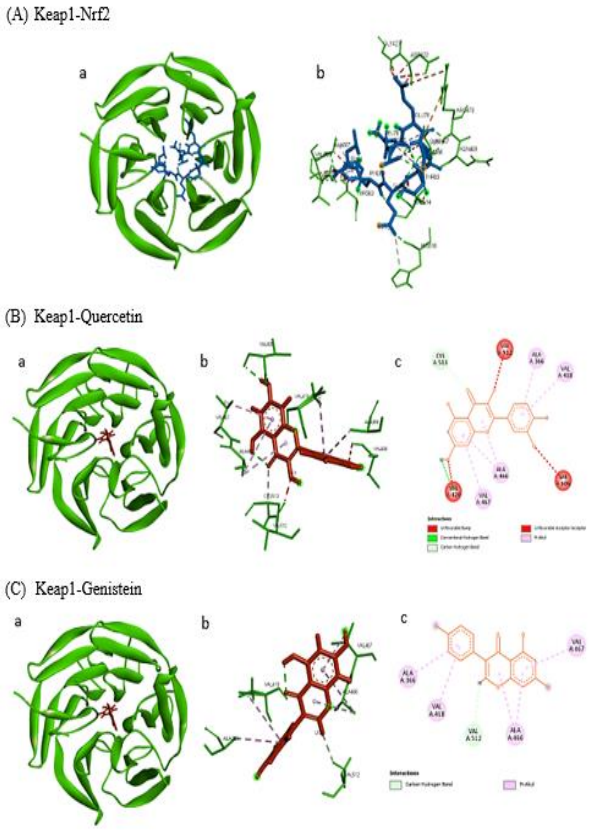


Figure 6: The 3D and 2D structure of the complex quercetin and genistein as Nrf2 - Keap1 interaction. Quercetin and genistein structure demonstrated in orange stick structure, Nrf2 is blue stick structure, and Keap1 showed green ribbon structures. a-b. 3D view, and c. 2D view.

Table 2: The interaction among Nrf2 and Keap1

Ligand-Protein complex	Binding energy (kJ/mol)	Residues (Distance in A)	Category	Types of Interaction
Keap1-Nrf2	-453.51	**A:VAL324:N (**3.02); **A:ARG470:NH1 (**5.27); **A:ARG470:NH2 (**5.40)	Electrostatic	Attractive Charge
		A:VAL467:HN (2.82); **A:ARG470:HN (**1.64); P:GLY81:O (2.97); **A:HIS516:HN (**1.44); **A:VAL608:HN (**2.81); P:GLU82:O (1.77); **P:GLU79:HN (**1.99); **P:THR80:HN (**2.97); **P:THR80:HN (**2.13); **P:GLY81:HN (**1.94); **P:GLU82:HN (**2.28); A:VAL514:O (3.01); **A:VAL324:CA (**2.32); **A:HIS516:CD2 (**3.64); **A:ALA466 (**4.88); **A:VAL467 (**4.49); A:ALA607 (4.25); A:VAL608 (5.22);	Hydrogen Bond	Conventional Hydrogen Bond
		A:ASP422:N (1.99); **A:ASP422:N (**2.12); **A:ASP422:CA (**2.19); **A:ASP422:HN (**1.42); **A:GLY423:N (**2.05); **A:GLY423:HN (**1.29); **A:VAL467:C (**2.32); **A:ASN469:HN (**1.59); **A:VAL514:CG1 (**2.14); **A:VAL514:CG1:B (**1.27); **A:VAL514:CG1:B (**1.57); **A:VAL514:CG1:B (**1.58); **A:VAL514:CG1:B (**2.14); **A:THR560:OG1 (**1.85); **A:THR560:OG1 (**0.99); **A:THR560:OG1 (**1.51); **A:THR560:HG1 (**1.27); **A:THR560:HG1 (**0.85); **A:ALA607:CA (**2.17); **A:VAL608:CB (**2.12);	Unfavorable	Unfavorable Bump
		P:GLY81:CA (1.12)	Unfavorable	Unfavorable Bump; Carbon Hydrogen Bond
		A:ASP422:OD1 (4.10); **P:ASP77:OD1 (**5.27)	Unfavorable	Unfavorable Negative-Negative

Note: bold lettering indicates donor residues/atom

Increased ROS can activate Nrf2 by producing anti-oxidative enzymes such as HO-1 and GCLC and increasing the Cu, Zn-SOD concentration in spermatogonia. These anti-oxidative enzymes positively produce endogenous enzymatic antioxidants, including catalase, SOD, GPx and non-enzymatic endogenous antioxidants, including glutathione [12].

Exogenous antioxidants act as free radical scavengers by increasing the natural defence system or endogenous antioxidants, thereby reducing the elevation of oxidative stress caused by excess free radicals [15]. The administration of *M. crenata* extract was able to increase endogenous enzymatic antioxidants or SOD1 in the testes. The current study revealed that SOD1 expression increased in the testes of rats given 86.4 mg/200 g of *M. crenata* extract. This could be due to quercetin's ability to break the bond between Keap1 and Nrf2. If Nrf2 does not bind to Keap1, it will move to the nucleus and induce SOD1 synthesis. Based on the in silico analysis, the bond position between Keap1 and quercetin was the same as the bond position between Keap1 and Nrf2. The same can be said for the Keap1-Genistein bond.

Flavonoids have a role as exogenous antioxidants that are directly scavenging free radicals. The mechanisms of flavonoids as an antioxidant is interacting with various antioxidant enzymes. Titisari et al [16] mentioned that other *M. crenata* extract compounds are isoflavones, a class of flavonoids. Astuti *et al* [17] reported that isoflavones act on the testes by acting as antioxidants. This was evidenced by the decreased formation of MDA and increased levels of Cu and Zn-SOD. The role of isoflavones in inhibiting oxidative stress correlates with high levels of SOD1. This is supported by Kini *et al* [18], which demonstrated an increase in SOD1 and glutathione levels in testes induced by Cadmium. High levels of Cu and Zn-SOD protect spermatogonia cells in the testes to reduce ROS levels and then prevent oxidative stress [19]. In this study, *M. crenata* supplementation induced a significant increase in the expression of Nrf2 and its downstream target genes SOD1 in the testes of the male rat, suggesting that *M. crenata* plays a role as a fertility agent due to its antioxidant properties.

Quercetin is a polyphenolic flavonoids compounds, which possess strong antioxidant

Table 3: Interaction among quercetin, genistein, Nrf2, and Keap1

Ligand-protein complex	Binding energy (kJ/mol)	Residues (Distance in A)	Category	Types of interaction
Quercetin - Keap1	-251.8	A:VAL420:O (2.09); **A:CYS513:CA (**3.24);	Hydrogen Bond	Conventional Hydrogen Bond
		A:ALA466 (4.76); A:ALA466 (5.22); A:VAL467 (4.63); A:ALA366 (4.01); A:VAL418 (5.02)	Hydrophobic	Pi-Alkyl
		A:VAL420:N (2.15); **A:VAL420:HN (**1.42);	Unfavorable	Unfavorable Bump
		A:VAL512:O (2.08)	Unfavorable	Unfavorable Bump
		A:VAL606:O (2.88)	Unfavorable	Unfavorable Acceptor-Acceptor
Quercetin-Keap1 +Nrf2	-441.1	**A:VAL324:N (**4.13); **A:ARG470:NH1 (**5.13)	Electrostatic	Attractive Charge
		A:VAL324:HT2 (2.58); **A:VAL608:HN (**2.60); **P:ASP77:HN (**1.77); **P:GLU79:HN (**2.00); **P:THR80:HN (**2.97); **P:THR80:HN (**2.13); **P:GLY81:HN (**1.94); **P:GLU82:HN (**2.28); **A:VAL324:CA (**2.35); **A:CYS368:CA (**2.82)	Hydrogen Bond	Conventional Hydrogen Bond
		P:ASP77:OD1 (4.69); **P:ASP77:OD1 (**3.79); **P:GLU78:OE1 (**4.76)	Electrostatic	Pi-Anion
		P:GLU78:HN (2.90);	Hydrogen Bond	Pi-Donor Hydrogen Bond
		A:VAL420 (5.35); **A:VAL467 (**4.93)	Hydrophobic	Alkyl
		A:CYS368:CB (1.94); **A:CYS368:CB (**2.02); **A:VAL418:CG2 (**1.88); **A:VAL418:CG1:B (**2.22); **A:VAL418:CG1:B (**1.84); **A:VAL418:CG1:B (**2.16); **A:VAL420:O (**2.05); **A:VAL420:CG2 (**2.37); **A:THR560:OG1 (**1.84); **A:ALA607:CB (**2.15)	Unfavorable	Unfavorable Bump
		A:THR560:HG1 (1.35);	Unfavorable	Unfavorable Bump;Conventional Hydrogen Bond
		P:ASP77:OD1 (5.27)	Unfavorable	Unfavorable Negative-Negative
Genistein - Keap1	-246.6	A:VAL512:O (2.58)	Hydrogen Bond	Carbon Hydrogen Bond
		A:ALA466 (4.66); A:ALA466 (5.32); A:VAL467 (4.27); A:ALA366 (4.61); A:VAL418 (5.23)	Hydrophobic	Pi-Alkyl
Genistein-Keap1+Nrf2	-425.3	A:VAL512:O (2.58); P:GLU79:OE2 (2.52);	Hydrogen Bond	Carbon Hydrogen Bond
		P:ASP77:OD1 (4.08); **P:ASP77:OD1 (**3.38); **P:GLU78:OE1 (**4.70)	Electrostatic	Pi-Anion
		A:ALA466 (4.66); A:ALA466 (5.32); A:ALA366 (4.61); A:VAL418 (5.23); A:VAL467 (4.27)	Hydrophobic	Pi-Alkyl
		P:GLU78:CG (2.17); P:GLU78:CG (1.44)	Unfavorable	Unfavorable Bump

activity by maintaining oxidative stress balance. Based on electronegativity, electron affinity, electrophilicity value, ionization potential, and hardness, quercetin serves as an electron donor rather than an electron acceptor. The antioxidant properties of quercetin are mostly attributed to OH groups in the B-ring and C-ring, although OH groups in the A-ring play a minor part in the hydrogen atom transfer (HAT), single electron transfer followed by proton transfer (SET-PT) and sequential proton loss electron transfer (SPLET) [20].

Veiko *et al* [20] found quercetin has a higher antioxidant capacity compared to naringenin and catechin. The powerful antioxidant activity of quercetin is due to the C2=C3 double bond in the C ring, the planar structure, and optimal HOMO (Highest Occupied Molecular Orbital) delocalization of quercetin [20]. The inhibitory effect of *M. crenata* extract on DPPH radical may be attributable to the C2=C3 double bond in the C ring of quercetin, which is the greatest compound in *M. crenata* extract.

Based on *in silico* analysis, quercetin can bind to SOD1 with a lower binding affinity, indicating that the bond is strong. The interaction between genistein and quercetin against SOD1 could increase the activity of SOD1. It was supported by flow cytometry data that found the level of SOD1 expression in rat testes significantly increased after receiving 86.4 mg/200 g BW of *M. crenata* extract (Figure 4). Furthermore, genistein-SOD1 and quercetin-SOD1 complexes induce the activity of glutathione (GSH) [21].

CONCLUSION

The findings of this study provide evidence that *M. crenata* leaf extract treatment results in the significant regulation of SOD1 expression via Nrf2 pathway in rat testes, possibly through high quercetin and genistein contents in its extract. Quercetin inhibits Nrf2-Keap1 interaction by substituting Nrf2 binding site as a native ligand. *M. crenata* extract can also be considered a natural source of antioxidants and phytoestrogen, and should be further investigated as a fertility agent and for the treatment of testicular dysfunctions.

DECLARATIONS

Acknowledgements

Not provided

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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