



Kaempferol attenuates spinal cord injury by interfering inflammatory and oxidative stress by targeting the p53 protein: a molecular docking analysis

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Abstract

Background Kaempferol (KMF) is a flavone reported to have anti-oxidant and anti-inflammatory activity.

Objective The present study screened the effect of KMF in the animal model of spinal cord injury (SCI).

Results KMF caused a significant inhibition of spinal cord injury mediated oxidative stress and also suppressed the inflammatory reactions. The treatment of KMF also inhibited the levels of p53, TGF- β 1 and COX-2 whereas a significant elevation in Bcl-2/Bax ratio was observed after the rats were treated with KMF. The in silico docking analysis suggested potential binding of KMF having lower energy with p53 confirming the potential target of KMF.

Conclusion The treatment of KMF exerted neuroprotective effect by improving the anti-oxidant status and inflammatory response. The significant spinal cord injury protective effect of KMF in rats was attributed by targeting p53 and Bcl-2/Bax ratio.

Keywords Spinal cord injury · Kaempferol · P53 · Oxidative stress · Inflammatory response

Introduction

Spinal cord injury (SCI) is a traumatic disorder of the spine characterized by a higher rate of disability with a higher incidence rate leading to poor life quality with economic burdens to the family of the patient (Liebscher et al. 2015). Modes of travel and transportation have increased the number of morbidities every year (Liebscher et al. 2015). The treatment approaches for SCI majorly include operative therapy along with drugs and rehabilitative approaches for improving the overall functionality of the affected subjects.

SCI is featured by two types of injuries termed to be Primary and Secondary (Duetzmann et al. 2015). The primary injuries are a result of early jerk or force direct or indirect on the spinal cord tissue (Suwanna et al. 2014). Secondary injuries occur majorly around the site of primary injuries accompanied by responses which include increased oxidative burden and levels of inflammatory mediators damaging tissues in the periphery of the injured site (Uckermann et al. 2015; Luo et al. 2015). Oxidative stress is one of the responses due to an imbalance in the antioxidant system of the body and the reactive oxygen species (Kubota et al. 2012). Inflammatory response is accompanied by the release of number of mediators of

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inflammation such as TGF- β 1, COX-2 and p53 (Kubota et al. 2012). Studies have focused on targeting the secondary injuries as they are more liable and susceptible to therapies which may include anti-oxidants and anti-inflammatory agent.

Recently, major progresses have been achieved in the treatment of SCI by treating and relieving the progression of injuries (Silva et al. (2014)). Currently, the treating approach for the early phase of SCI includes surgical intervention along with a high dose of steroids such as Methylprednisolone. The surgical approach is done to decompress the injured spinal cord disc to a normal state (Ahuja et al. 2016) followed by therapy of Methylprednisolone. Though Methylprednisolone has a number of gains in treating SCI patients which include increased blood flow in the spinal cord and decreasing inflammatory response (Suberviola et al. 2008), however, the steroidal drug is associated with disadvantages of causing bleeding in GI tract, increased GI motility and causing secondary infections (Hurlbert et al. 2015). Hence there is an urgent need for molecules which would be safe in treating SCI.

Bioactive compounds have always been an effective natural source in the process of drug development. Traditional Chinese medicine (TCM) from many decades has been effectively used in clinical practice in China and in many parts of the world. TCM is reported to play an important part in the development of alternative medicine and hold the edge in treating SCI as number of experimental studies have been reported (Liu et al. 2015; Boots et al. 2008; Manach et al. 2005). Though it is understood that TCM cannot replace the surgical management of SCI, still it could play a very important factor in treating spinal cord injury. Number of bioactive molecules have been studied and have been reported in the management of SCI Zhang et al. (2017). Among the bioactive compounds, flavonoids are the most studied compounds for their anti-inflammatory, anticancer and neuroprotective effects in number of studies, also this group of compounds are reported to exert a protective effect in SCI Zhang et al. (2017). Kaempferol (Fig. 1) is a natural flavonoid found in a number of plants and has been found to possess antioxidant and anticancer activity (Oliveira et al. 2002; Wang et al. 2018). In addition to this Kaempferol has been reported to show neuroprotective, anti-inflammatory, anti-oxidant and anti-diabetic activity (Arif et al. 2018; Li et al. 2017; Nascimento et al. 2017; Suchal et al. 2017; Wang et al. 2006; Wu et al. 2016). Also, foods containing Kaempferol have shown ameliorating effect in cancers of skin, liver and colon (Pei et al. 2017; Kocic et al. 2013; Calderón-Montaña et al. 2011). Being a flavonoid of great pharmacological benefits reports investigating the role of Kaempferol in SCI are missing. In the present investigation, we screened the

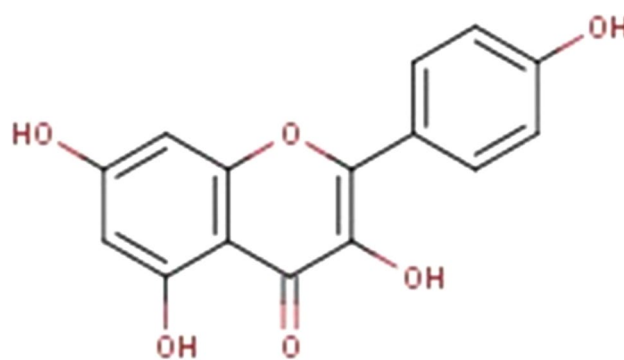


Fig. 1 Chemical structure of Kaempferol

role of Kaempferol in SCI and also studied the involved mechanism.

Materials and methods

Materials, animals and groupings

For the study, Kaempferol was obtained from Sigma-Aldrich USA and was used without any further purification. For animal studies Sprague Dawley rats aging between 7 and 8 weeks were selected weighing between 210 and 220 g, the animals were housed in polypropylene cages at 25°C and submitted to the dark–light cycle of 12 h. The animal studies were approved by the ethical review board of The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China, the approval number for the study was S0144C, and the experiments adhered to Regulations for the Administration of Affairs Concerning Experimental Animals, PRC China. The rats were divided into 5 groups randomly, Group 1: SCI injury rats ($n=12$), Group 2: Sham-operated rats ($n=12$), Group 3: KMF-5 (5 mg/kg/day) ($n=12$), Group 4: KMF-10 (10 mg/kg/day) $n=12$, Group 5: KMF-25 (25 mg/kg/day) $n=12$. The SCI rat model was created by inducing injury at thoracic 12 level, the rats were anesthetized with ketamine and xylazine (80 and 10 mg/kg respectively) and laminectomy was carried out, the muscle and skin at the 12 thoracic level of the spinal cord were cut. The injury was induced by the weight drop method using the weight of moderate intensity using an impactor. The sham-operated rats were not submitted to weight drop injury. The KMF treated rats received the treatment for five days.

Histopathological studies

The spinal cord tissues were collected on the 6th day, the rats were euthanized by injecting pentobarbital (200 mg/ml) and the spinal cord tissues were isolated. The tissues were fixed in paraformaldehyde (4%) for 24 h and then were fixed

using paraffin to form blocks, these blocks were submitted to rotary microtome for obtaining sections of 4 μm thickness. The sections were dewaxed with the help of xylene and then treated with ethanol. The tissue sections were stained using hematoxylin and eosin (H and E).

Locomotor activity by Basso-Beattie-Bresnahan (BBB) scoring and content of water in spinal cord tissues.

The locomotor activity was evaluated by BBB scoring scale ranging from 0 to 20. The BBB scoring took into account about the movements of hind limbs. The score of '0' indicated no limb movement and score of '20' suggested normal locomotor activity. After the rats received treatments of KMF, the spinal cord tissues were collected and weighed which represented wet weight of tissue, after this the tissues were dried at 80 °C for 48 h and weighed again representing the dry weight of spinal cord tissues. The total water content of spinal cord tissue was accounted as a ratio of dry weight/wet weight.

ELISA assay

The spinal cord tissues were homogenated using a tissue homogenizer and the homogenates were then centrifuged at 5000 g for 15 min in a cooling centrifuge at 4 °C, the protein levels were analyzed in the supernatants with a protein estimation kit. The supernatant was analyzed for superoxide dismutase (SOD), Catalase (CAT), Malondialdehyde (MDA) and glutathione peroxidase (GSH) using the respective ELISA assay kits. The supernatants were also processed to analyze contents of tumor necrosis factor NF- κ B p65, (TNF)- α , IL-6 and interleukin (IL)-1 β with the help of ELISA kits. The optical density was observed at 405 nm.

Quantitative reverse transcription-quantitative polymerase chain reaction (qRT-PCR).

The spinal cord tissues were processed as per the supplied procedure using TRIzol reagent for isolating RNA. About 1 μg of RNA was used to synthesize cDNA with the help of a RT-PCR kit (ThermoFisher USA). The gene-level expression of TGF- β 1 and cyclooxygenase (COX-2) was done by qRT-PCR system (ThermoFisher USA). The programming of the system included 25 cycles at 95 °C hold time of 15 s then annealing at 60 °C hold time of 20 s and finally stretching to 70 °C following 15 s hold. The primers utilized were for TGF- β 1 F, 5'-AGG GCT ACC ATG CCA ACT TC-3' and R, 5'-CCA CGT AGT AGA CGA TGG GC-3', COX-2 forward, TTC CAA TCC ATG TCA AAA CCG T and reverse, AGT CCG GGT ACA GTC ACA CTT and β -actin forward, GGC TGT ATT CCC CTC CAT CG and

reverse, CCA GTT GGT AAC AAT GCC ATG T. Quantitative analysis was performed by ΔCt method. The quantification was based on normalized Ct deviation of target genes compared to the control. The Ct value is the point of crossing of threshold whereas the ΔCt is the point of crossing for sample and control.

Western blot studies

The tissues of the injured spinal cord were homogenized, the homogenates were centrifuged at 500 g for 15 min and supernatants were analyzed for total protein content. About 50 μg of proteins were submitted to SDS-PAGE membrane (12%) and then transferred to PVDF membrane (ThermoFisher USA). The resultant membranes were incubated with 5% skimmed milk for 1 h at room temperature and incubated for 12 h at 4 °C along with antibodies against Bax, Bcl-2, p53 for the same actin was selected as a loading control. The resultant membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody for 60 min at 25 °C. The blots were quantified by the densitometry method.

In silico docking analysis

For studying the in silico target interaction we performed homology modeling for p53 subunit and the ligand Kaempferol. The protein crystal structure was downloaded from the protein database. The homology modeling was done by performing the SWISS modeling for p53. The docking was done by Autodock Vina tools 1.5.7 (The Scripps research institute). The interaction between protein and ligand was viewed by PyMol 2.4.0 (Schrodinger LLC), ACD.Chem-Sketch (acdlabs).

Preparation of ligand and protein for molecular docking

The ligand and protein were prepared for docking with the help of Autodock Vina, for the same the ligand was prepared by adding hydrogens and removing water molecule, Kollman charges were also added and at last the PDB format file was saved. The protein structure was also prepared by removing the water molecule, adding hydrogen's and charges. The grid map was formulated with the help of Auto grid 4, for the same a grid box was prepared. The coordinates and dimensions of the grid box for the x, y and z coordinates for the protein p53 subunit were – 1.373, 10.108 and 20.024, respectively, the sizes of all the three coordinates was 20, respectively. For minimizing and optimizing the energies we selected the Lamarckian genetic algorithm for the docking simulation.

Statistical analysis

All the results were presented as means \pm standard deviation, the computation was done with GraphPad Prism software (version 6). For statistical analysis, Spearman's rank correlation and Mann–Whitney *U* test were performed. The value $p < 0.05$ was confirmed to be an indicator of significance.

Result

Kaempferol ameliorated spinal cord injury in rats and improved locomotor activity

The rats induced with SCI and treated with vehicle showed histology with significant injury compared to sham-operated rats. The rats treated with KMF at the dose 10 and 25 mg/kg/day showed signs of improvement in the histology of spinal cord tissue with significantly less damage compared to non-treated SCI rats (Fig. 2a).

Effect of SCI on locomotor activity was assessed by opting BBB scoring method; the results suggested that the average BBB scores were lowest in the SCI rats treated with vehicle whereas the scores were highest in Sham-operated rats (Fig. 2b). After receiving the treatment of KMF at the dose 10 and 25 mg/kg/day the rats showed significant improvement in BBB scoring compared to vehicle-treated rats (Tween-20 1%), however dose of 5 mg/kg/day do not show significant changes (Fig. 2b).

Kaempferol decreases spinal tissues water content in spinal cord injured rats

The injured spinal cord tissues were examined for water content in SCI rats, the content was analyzed after the SCI rats were treated with KMF. It was found that the SCI rats treated with vehicle showed increased water content in the isolated spinal cord tissue compared to sham-operated rats (Fig. 3). On the 5th day the water content in the injured spinal cord tissues of rats treated with 10 and 25 mg/kg/day dose of KMF was decreased in SCI rats compared to vehicle-treated rats (Fig. 3).

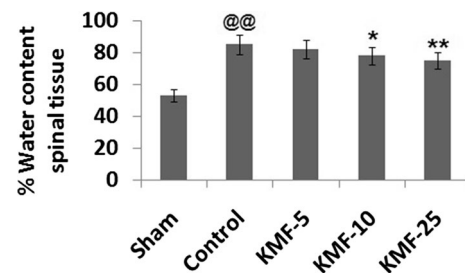


Fig. 3 KMF decreases the total water content in the spinal cord tissues of SCI rats. The SCI rats treated with KMF at a dose of 10 mg/kg (KMF-10) and 25 mg/kg (KMF-25) showed decreased total water content compared to control rats. @@ $p < 0.01$ compared to sham-operated rats, * $p < 0.05$ compared to control rats, ** $p < 0.01$ compared to control rats

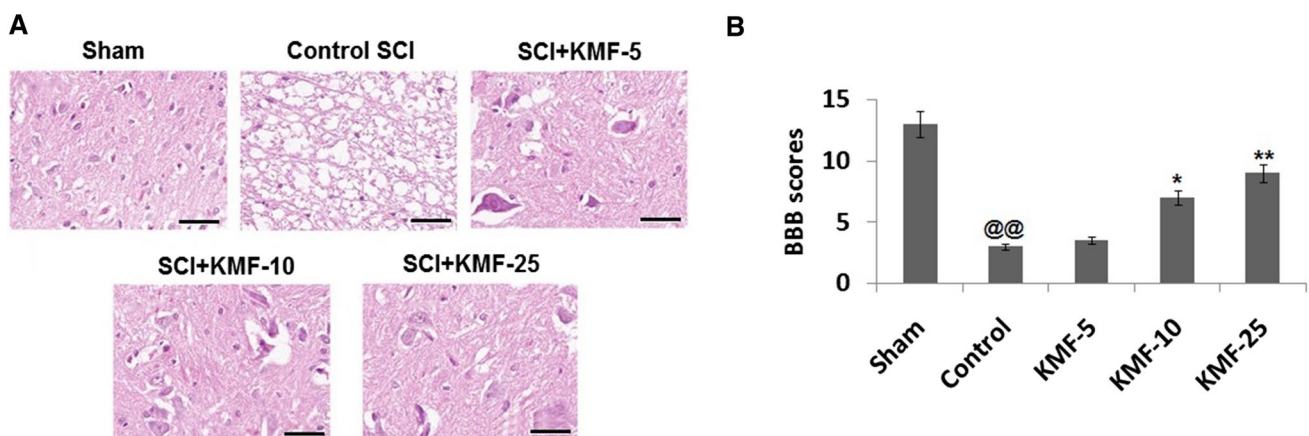


Fig. 2 Kaempferol attenuates the histopathology in the injured spinal cord tissue and improve locomotor activity in SCI rats. **a** histopathology of spinal cord tissue submitted to injury followed by treatment of KMF at selected doses. The spinal cord tissue of KMF treated rats at a dose of 10 mg/kg (KMF-10) and 25 mg/kg (KMF-25) showed

signs of improvement. **b** KMF improved the BBB scoring in SCI rats receiving treatment of KMF (10 and 25 mg/kg). @@ $p < 0.01$ compared to sham-operated rats, * $p < 0.05$ compared to control rats, ** $p < 0.01$ compared to control rats

Kaempferol improves antioxidant status and decreases oxidative stress in SCI rats

Effect of treatment of KMF on the status of antioxidant enzymes and levels of Malondialdehyde (MDA) was studied in SCI rats. In SCI rats it was observed that the levels of MDA which is marker of lipid peroxidation were increased whereas the activities of antioxidant enzymes i.e., (Superoxide dismutase) SOD, (Glutathione peroxidase) GSH-PX and Catalase (CAT) were suppressed suggesting increased oxidative stress in SCI rats (Fig. 4). However, the SCI rats when were treated with KMF (10 and 25 mg/kg/day) showed improvement in oxidative stress burden compared to vehicle-treated control rats. It was observed that the MDA levels were decreased and activity of SOD, GSH-PX and CAT were increased suggesting improved status of anti-oxidant enzymes. However, the dose of 5 mg/kg/day does not produce any significant effects on oxidative stress (Fig. 4).

Kaempferol shows anti-inflammatory effect in SCI rats

To study the effect that KMF shows anti-inflammatory effect in SCI rats we evaluated the expression of markers of inflammatory response i.e., IL-1 β , IL-6, NF- κ B p65 and TNF- α were studied by performing ELISA assay. As the outcomes

suggested (Fig. 5a), the SCI rats showed increased levels of IL-1 β , IL-6, NF- κ B p65 and TNF- α compared to sham-operated rats. It was observed that treatment of KMF (10 and 25 mg/kg/day) inhibited the levels of IL-1 β , IL-6, NF- κ B p65 and TNF- α significantly compared to untreated SCI rats (Fig. 5a).

To screen the role of KMF on levels of COX-2 in spinal cord injured rats we performed qRT-PCR studies for mRNA expression of COX-2 in experimental SCI rats. The outcomes suggested that the SCI rats showed over-expression of COX-2 mRNA levels against sham-operated rats (Fig. 5b). In SCI rats treated with 10 and 25 mg/kg/day dose of KMF caused significantly decreased levels of mRNA COX-2 in SCI rats compared to vehicle-treated SCI rats (Fig. 5b).

Kaempferol inhibits the TGF- β 1 mRNA levels in spinal cord injured rats

To evaluate the role of KMF on levels of TGF- β 1 in rats submitted to SCI, we performed qRT-PCR study for detecting the mRNA expression levels of TGF- β 1. It was observed that the mRNA TGF- β 1 levels were significantly increased in SCI rats compared to the sham-operated rats (Fig. 5c). In SCI rats treated with 10 and 25 mg/kg/day dose of KMF demonstrated significantly decreased mRNA TGF- β 1 levels in SCI rats compared to vehicle-treated rats (Fig. 5c).

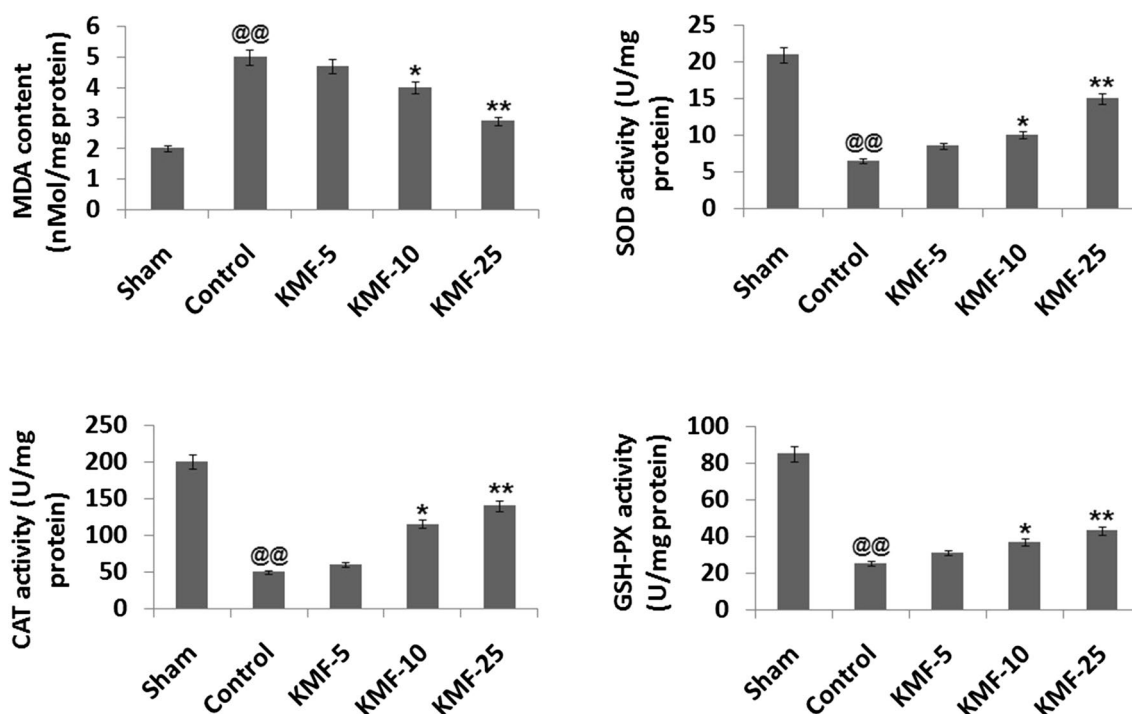


Fig. 4 KMF shows anti-oxidant activity in rats submitted to SCI. The SCI rats treated with KMF at a dose of 10 mg/kg (KMF-10) and 25 mg/kg (KMF-25) showed decreased levels of MDA and significant

improvement in the activity of antioxidant enzymes SOD, CAT and GSH-PX. @@ p < 0.01 compared to sham-operated rats, * p < 0.05 compared to control rats, ** p < 0.01 compared to control rats

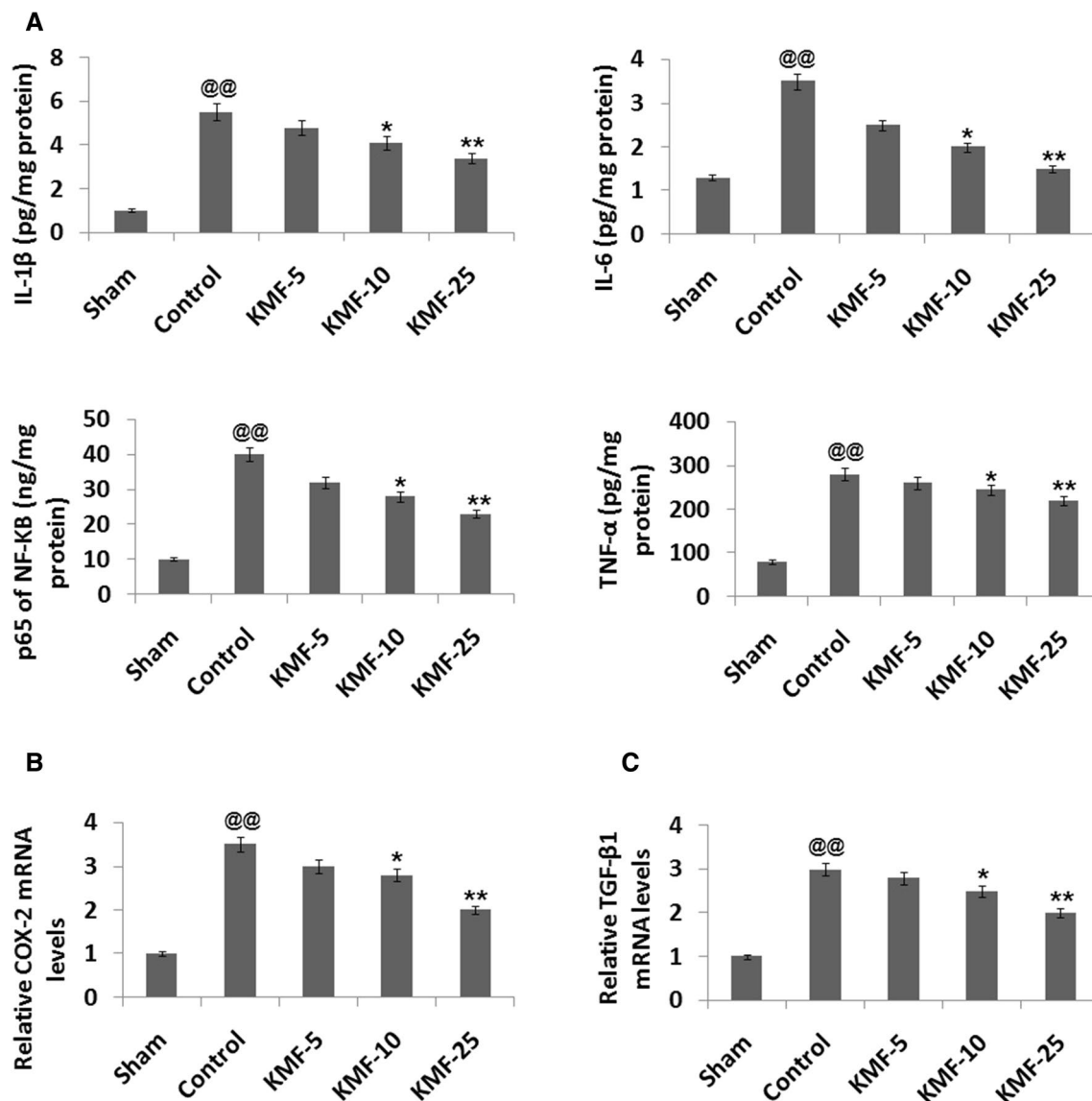


Fig. 5 KMF shows anti-inflammatory activity and ameliorates levels of mRNA COX-2 and TGF-β1 levels in SCI rats. **a** The treatment of KMF at a dose of 10 mg/kg (KMF-10) and 25 mg/kg (KMF-25) showed significant improvement in expression levels of various mark-

ers of inflammation in SCI rats compared to the control group. **b** The treatment of KMF improved the mRNA levels of COX-2 (**b**) and TGF-β1 (**c**). @@ $p < 0.01$ compared to sham-operated rats, * $p < 0.05$ compared to control rats, ** $p < 0.01$ compared to control rats

Kaempferol suppresses p53 expression and Bcl-2/Bax ratio in spinal cord injured rats

To study the involvement of KMF on expression levels of p53 in rats submitted to spinal cord injury, we evaluated the protein levels of p53 by western blot analysis. The expression levels of p53 were found to be on a lower side in SCI rats compared to sham-operated rats (Fig. 6a). In the SCI rats which were treated with 10 and 25 mg/kg/day dose of KMF had increased levels of p53 compared to SCI rats which were treated with vehicle only (Fig. 6a).

The western blot assay of Bcl-2 and Bax was done and the ratio of Bcl-2/Bax was measured. It was observed that the SCI rats had a high ratio of Bcl-2/Bax against sham-operated rats, however, the treatment of 10 and 25 mg/kg/day dose of KMF caused a significant suppression in the ratio compared to vehicle-treated SCI rats (Fig. 6b).

p53 was the favorable target for Kaempferol

Looking into the results of in vivo model In silico docking analysis was undertaken by preparing the ligand Kaempferol

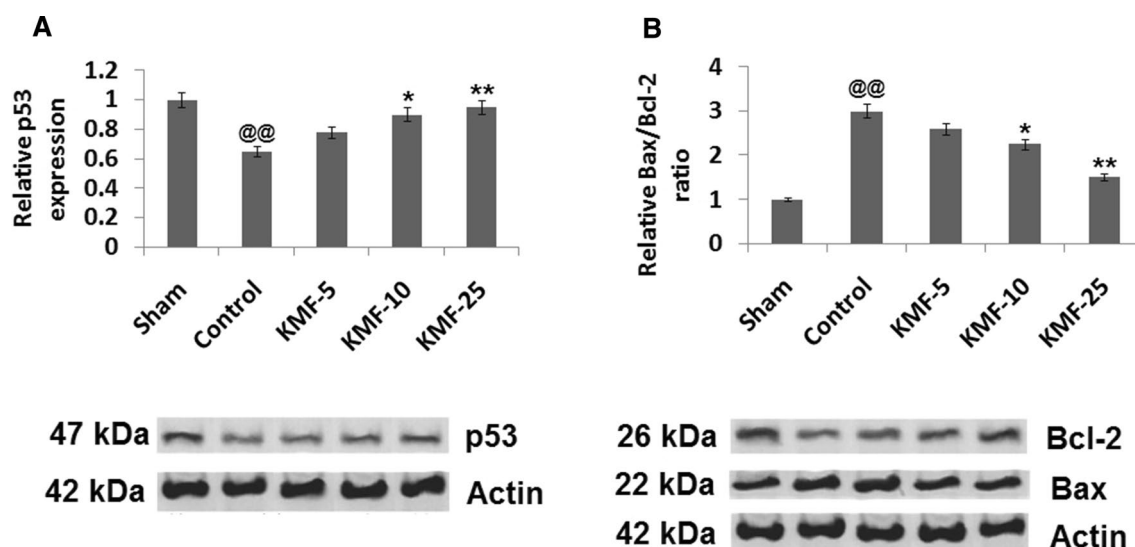


Fig. 6 KMF inhibits the expression of p53 and ratio of Bcl-2/Bax in SCI rats. Treatment of KMF at a dose of 10 mg/kg (KMF-10) and 25 mg/kg (KMF-25) showed significant improvement in expression

levels of (a) p53 and (b) ratio of Bcl2/Bax in SCI rats. @@ $p < 0.01$ compared to sham-operated rats, * $p < 0.05$ compared to control rats, ** $p < 0.01$ compared to control rats

Table 1 Docking scores for protein p53 with the ligand molecule KMF

Docking score for p53 protein and KMF			
Mode of ligand	Affinity (kcal/mol)	Distance from rmsd l.b	Best mode rmsd u.b
1	− 7.4	0.00	0.00
2	− 6.4	11.750	16.722
3	− 5.2	10.534	14.771
4	− 4.5	4.126	6.622
5	− 4.1	11.542	17.118
6	− 4.0	12.886	16.112
7	− 3.0	11.118	13.149
8	− 2.9	12.701	14.730
9	− 2.8	11.803	15.105

and protein p53. The scores of docking by simulation suggested potential binding of protein p53 with ligand Kaempferol. The results of binding energies clearly showed that ligand Kaempferol had low binding energies after binding with p53 (Table 1). The solid surface view and ribbon view showed potential binding of ligand with the protein p53 (Fig. 7).

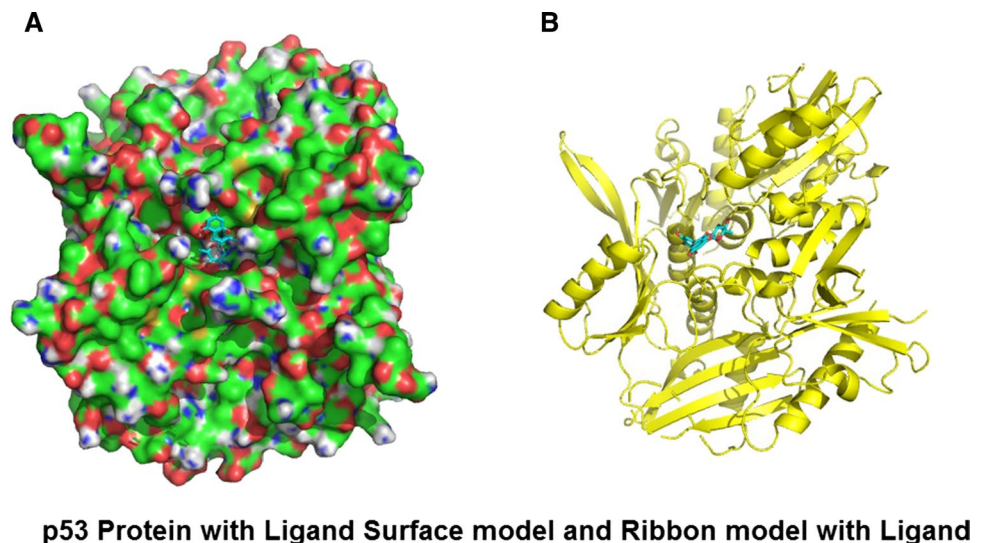
Discussion

Spinal cord injury is divided majorly into acute and chronic spinal cord injury (Due et al. 2014). The major managing approaches for countering SCI include surgical intervention

along with drug therapy immediately after SCI (Dulin et al. 2013). Though surgeries being the major correcting option, the outcomes are not certain, hence number of patients are affected by neurological deficits and suffer a lifelong disability. Looking into this there is an urgent need for therapies which along with a surgical approach could be helpful in treating SCI. In the present work, we screened a bioactive molecule KMF, a flavone for its activity in spinal cord injury rats; it was found that KMF at 10 and 25 mg/kg/day improved the locomotor activity as seen in BBB scoring and decreased the spinal cord water content isolated from spinal cord injured rats. The treatment of KMF significantly decreased the levels of MDA which is a marker of lipid peroxidation and oxidative stress; the treatment also significantly improved the anti-oxidant enzymes activity of SOD, CAT and GASH-PX demonstrating the anti-oxidant activity of KMF. The study also demonstrated that the treatment of KMF in SCI rats ameliorated the expression levels of markers of inflammatory response i.e., IL-1 β , IL-6, NF- κ B p65 and TNF- α suggesting anti-inflammatory potential of KMF. Earlier it was reported that KMF ameliorated oxidative stress in streptozotocin-induced diabetic rats Al-Numair et al. 2015. Also in a study, treatment of KMF resulted in inhibition of multiple mediators of inflammation in rats (Bian et al. 2019). The findings of the present study were in agreement with the findings of these studies.

Increased burden of free radicals after suffering from SCI target postsynaptic neurons and also trigger the adjacent astrocytes and microglial cells (Brown and Sawchenko 2007). Oxidative stress after SCI leads to altered levels of free radical species destabilizing the homeostasis both

Fig. 7 In silico docking analysis showing Interaction between Ligand (Kaempferol) and Protein (p53) **a** Surface model and **b** Ribbon model



inside and outside the cell membranes (Fu et al. 2014). It was found that excessive infiltration of Calcium in mitochondria resulted in its destruction (Siddiq et al. 2009). Earlier it was reported that free radicals could decrease the levels of GSH whereas the free radicals scavengers had an inverse effect, in addition to this free radicals increased the release of inflammatory cytokines such as TNF- α (Wang et al. 2015). Here in our study, treatment of KMF (10 and 25 mg/kg/day) resulted in significantly improved oxidative stress and also inflammatory mediators.

Upregulation of COX-2 is linked with increased density of micro-vessels Yoon et al. (2010). In addition to this COX-2 is associated with the process of angiogenesis Zani et al. (2014). It is documented that the mesenchymal stem cells present in the bone marrow are responsible for the release of various inflammatory cytokines which further facilitate angiogenesis in CNS He et al. (2014). In the current study, it was evidenced that the dose of KMF 10 and 25 mg/kg/day resulted in significant inhibition of COX-2 as well as TGF- β 1 mRNA levels in SCI rats, the results clearly suggested anti-inflammatory effects of KMF.

Increased levels of p53 result in apoptosis or influences the apoptosis-related genes (Kim et al. 2015). Expression of p53 at transcriptional levels leads to activation of WAF and G1P1 genes which further suppresses the activity of cyclin-dependent kinase (Navrkalova et al. 2013). The inhibition of p53 gene suppresses the levels of Bcl-2 and also inhibits its functioning (Kotipatruni et al. 2011), p53 is considered as the direct agonist of Bax and which leads to increased protein levels of Bax which also alters the Bcl-2/Bax ratio and leads to apoptosis Lee et al. (2015). Here the treatment of KMF resulted in significant suppression of p53 levels and also inhibited the ratio of Bax/Bcl-2 in spinal cord injured rats. The outcomes suggested that KMF ameliorated oxidative stress-mediated damage via regulating p53 levels and Bax/Bcl-2 ratio in SCI rats. Further the results of in silico

docking analysis suggested that p53 had potential binding with KMF, confirming the other findings of the study.

In conclusion, the present work confirmed that treatment of KMF improved the BBB score and improved the histology of spinal cord tissues in SCI rats via ameliorating oxidative stress and inflammatory response involving the NF- κ B p65, COX-2, Bax/Bcl-2 and TGF- β 1. Docking analysis confirmed the potential interaction between KMF and p53. However, a detailed experimental study is still needed to screen the events associated with KMF in improving the homeostasis following SCI.

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Author's contributions All the authors contributed equally in preparing the manuscript. LC and KC planned the study. YG, CL, WM, WZ, JZ, HZ along with LC and KC performed the experiments and formatted the data. All the authors contributed to preparing the manuscript and reviewed the paper before submitting.

Declarations

Conflict of interest Lu Chen, Kai Cao, Yurong Gu, Chao Luo, Wei Mao, Weijun Zhou, Jinwei Zhu, Huiying Zhang authors have no conflict of interest.

Ethical approval The animal studies were approved by the ethical review board of The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China, the approval number for the study was S0144C.

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