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Syringic acid demonstrates an anti-inflammatory effect via modulation of the NF-κB-iNOS-COX-2 and JAK-STAT signaling pathways in methyl cellosolve-induced hepato-testicular inflammation in rats

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ABSTRACT

Syringic acid (SACI) is an emerging nutraceutical and antioxidant used in modern Chinese medicine. It has potential neuroprotective, anti-hyperglycemic, and anti-angiogenic properties. Methyl cellosolve (MCEL) has been reported to induce tissue inflammation in the testis, kidney, liver, and lung. This study aimed to investigate the effect and probable mechanism of action of SACI on MCEL-induced hepatic and testicular inflammation in male rats. Compared to the control group, administration of MCEL to rats significantly increased the levels of IL-6, TNF-α, iNOS, COX-2, and NF-κB in the liver and testis. Additionally, the total mRNA expressions of JAK1 (in the liver only), STAT1, and SOCS1 were significantly increased in both the liver and testis, while testicular JAK1 total mRNA levels were significantly decreased.

The expression of PIAS1 protein was significantly higher in the liver and testis. Treatments with SACI at 25 (except liver iNOS), 50, and 75 mg/kg significantly decreased the levels of IL-6, TNF-α, iNOS, COX-2, and NF-κB compared to the control group. Furthermore, the total mRNA expressions of JAK1 and SOCS1 in the liver were significantly reduced by all doses of SACI investigated, while the total mRNA levels of liver and testis STAT1 were significantly reduced by 25 and 50 mg/kg of SACI only. In the testis, the mRNA level of SOCS1 was significantly reduced by all doses of SACI compared to MCEL only. Additionally, SACI (at 75 mg/kg) significantly reduced PIAS1 protein expression in the liver, while in the testis, SACI at all investigated doses significantly reduced the expression of PIAS1. In conclusion, SACI demonstrated a hepatic and testicular anti-inflammatory effect by inhibiting the MCEL-induced activation of the NF-κB and JAK-STAT signaling pathways in rats.

1. Introduction

Glycol ethers are widely used chemical compounds in various industries for producing printed circuit boards, coatings, inks, dyes, jet fuel deicers, pesticides, photography, plasticizers, and herbicides. Methyl cellosolve (MCEL), a member of the EGEs, primarily attacks cells with high metabolic rates that can divide rapidly, such as testicular epithelium germ cells, thymus, bone marrow, and spleen [1–3]. MCEL toxicity can interfere with the behavioral, physiological, and biochemical functions in humans and animals, leading to disorders of the heart, brain, kidney, hematopoietic system, liver, and gonads [4–6]. In rabbits, MCEL-induced testicular damage was first reported by Wiley et al. [7] and has been confirmed by many researchers [2,8–10]. MCEL is the most harmful alkyl ether, which can destroy the testes of rats (Watanabe et al., 2000), affecting the quality and concentration of sperm in mice, rats, and rabbits [8]. Additionally, it can impair mating performance and sperm characteristics in the epididymis of rats [11].

The antioxidant activity of phenolic compounds is attributed to their ability to function as hydrogen donors, reducing agents, metal chelators, and free radical scavengers, which help to prevent different types of human ailments such as cancer, atherosclerosis, and hypertension

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Abbreviations

MCEL	methyl cellosolve
SACI	syringic acid
IL-6	interleukin 6
TNF-α	tumor necrosis factor alpha
iNOS	inducible nitric oxide synthase
COX-2	cyclooxygenase 2
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
SOCS1	suppressor of cytokine signaling
JAK1	Janus kinase
STAT1	signal transducer and activator of transcription
PIAS	protein inhibitor of activated STAT
IKK	IκB kinase
EGEs	ethylene glycol ethers
ELISA	enzyme-linked immunosorbent assay
RT-PCR	reverse transcriptase-polymerase chain reaction
cDNA	complementary DNA
mRNA	messenger RNA
DAB	3,3-diaminobenzidine
BSA	bovine serum albumin
SD	standard deviation
PGs	prostaglandins
AA	arachidonic acid

[12–14]. Syringic acid (SACI) is a natural plant compound that has gained wide attention in modern Chinese medicine (MCM). Radix isatidis (Banlangen) and Isatis indigotica, two Chinese herbal medicines that are rich in SACI, have been reported to possess various pharmacological properties, including anti-viral effects [15]. Other herbs used in modern Chinese Medicine where SACI has been located are Herba dendrobii [16], Lentinula edodes [17], Alpinia calcarata Roscoe leaves [18], and cereal grains [17]. Furthermore, SACI has demonstrated a plethora of pharmacological effects such as antiproliferative [19], strong antioxidant [20–22], anti-cancer [23], hepatoprotective [24], anti-inflammatory [20], antifibrotic [20], antiendotoxic [25], and anti-apoptotic [20]. The antioxidant effect of SACI has been recently reported to be via the modulation of the Nrf2-Keap signaling pathway, which mediates the restoration of the endogenous antioxidant system in the liver and testis of rats [22,26]. Syringic hydrazone, a synthetic analog of SACI, was reported to possess a double role of carbonyl and radical scavenging activities, thus preventing carbonyl and oxidative stress in atherosclerosis [27]. SACI obtained from Tamarix aucheriana has been shown to exhibit chemo-sensitizing and antimitogenic activities in colorectal cancer cells of humans by interfering with the proteasome activities, NF-κB- DNA binding, cell migration, apoptosis regulation, and cell-cycle arrest [28]. SACI's anti-diabetic property might be due to its ability to increase the release of insulin from the β-cells of the pancreas or its ability to augment the utilization of glucose by peripheral tissues. SACI's ability to downregulate aldose reductase expression and the structural proteins in the lens at the gene level has been demonstrated [29]. SACI has also shown anti-steatotic, anti-obesity, and anti-inflammatory effects by regulating the various genes participating in inflammation and lipid metabolism [30]. Again, the expressions of caspase-3 and beclin-1 protein in degenerated and dead motor neuronal cells of the spinal cord have been reported to be decreased by SACI [31], while it also lowered blood pressure and protected against organ destruction in hypertensive rats [32].

Cytokines are small cellular proteins that oversee and mediate or regulate inflammatory responses using complex cellular networks, and they are used as tissue biomarkers for understanding various diseases [33]. iNOS is one of the three known nitric oxide synthases. While the other two (nNOS and eNOS) are constitutively expressed, iNOS is induced in cells by pro-inflammatory cytokines [34]. Overproduction of NO due to dysregulation or overexpression of iNOS can lead to cellular toxicity that is associated with various human ailments, such as cancers, cardiac dysfunction, diabetes, and pain [35]. PGs are major inflammatory mediators, and some of them have been known to be actively produced during inflammation, which includes PGE2, PGI2, PGD2, PGF2α, and TXA2 [36]. The JAK-STAT signaling pathway is used as a mode of transducing signals intracellularly in response to growth hormones or cytokines [37]. SOCS proteins serve as negative-feedback inhibitors of signaling initiated by cytokines that operate through the JAK/STAT pathway [38]. These proteins operate as ubiquitin ligases, using Cullin5 as the ubiquitinating agent to ubiquitinate signaling components. There are eight members of the SOCS family, but SOCS1 is the most potent member of the family and is capable of inhibiting JAK directly [38].

Previous studies have reported the involvement of MCEL in the induction of hepatic [39], testicular [40], pulmonary [41], and renal [42] inflammation in rats. The mechanism of MCEL-induced tissue inflammation has not been fully elucidated. Based on the pharmacological credentials of SACI mentioned above, we investigated its anti-inflammatory mechanism of action in MCEL-induced hepato-testicular inflammation in male Wistar rats.

2. Materials and methods

2.1. Chemicals, primers, polyclonal antibody, and kits

MCEL ($C_3H_8O_2$), which has a purity of 99.5% and a CAS# of 109-84-4, is manufactured by BDH Laboratory Supplies, England. SACI ($C_9H_{10}O_5$ and 98% pure) was produced by AK Scientific, USA. The DNA primers for SOCS1, STAT1, and JAK1 were products of ShineGene Corporation, Shanghai, China. The rabbit polyclonal antibody for PIAS1 was produced by Cloud-Clone Corp, TX, USA. Rat-specific ELISA kits for NF-κB, iNOS, and COX-2 were produced by Elabscience Biotechnology Inc., Houston, TX, USA, while rat-specific IL-6 and TNF-α ELISA kits were produced by BioLegend, Beijing, China. All chemicals were purchased from certified outlets and were of analytical grades.

2.2. Rats and experimental design

Wistar rats (sex = male; n = 30; average weight = 220 g) were purchased from a rearing farm at the University of Ibadan, Ibadan, Nigeria. They were acclimatized for four weeks before the study commenced, and throughout the study, the animals had free access to food and water. Approval to carry out this study was granted by the local Institutional Animal Care and Use Committee (IACUC) of Afe Babalola University, Ado-Ekiti (ABUAD), with approval number ABUADREC 887359 – IDRD2021/062. After acclimatization, the rats were separated into six groups, each containing five rats, and were administered the test substances as shown in Table 1 below.

Table 1
The study design.

Group	Test substance administered	Duration
Group I	Control rats; were given food and water only	30 days
Group II	100 mg/kg of MCEL only	30 days
Group III	100 mg/kg of MCEL + 25 mg/kg of SACI simultaneously	30 days
Group IV	100 mg/kg of MCEL + 50 mg/kg of SACI simultaneously	30 days
Group V	100 mg/kg of MCEL + 75 mg/kg of SACI simultaneously	30 days
Group VI	75 mg/kg of SACI	30 days

All administrations were done orally by gavage for 30 consecutive days. Previous studies have concluded that SACI at 50 mg/kg yielded a better effect than 100 mg/kg [43,44]. Thus, we decided to test the effect of not only 50 mg/kg of SACI but also 25 mg/kg of SACI (a dose below 50 mg/kg) and 75 mg/kg of SACI (a dose above 50 mg/kg).

2.3. Sacrifice, sample collection, and preparations

On the 31st day, which was 24 h after the final administration, the rats were humanely sacrificed by cervical dislocation following international guidelines for the handling and utilization of experimental animals [45]. The liver and testis samples were dissected and rinsed with normal saline to remove any blood. A portion of the right liver and testis samples was then homogenized in freshly prepared 0.1 M (pH 7.4) phosphate buffer. The resulting homogenates were placed in 10 mL plain tubes and centrifuged at 5000 rpm for 10 min. The resulting supernatants were separated into 1 mL Eppendorf tubes for the quantification of hepatic and testicular levels of NF-κB, iNOS, COX-2, IL-6, and TNF-α using ELISA kits. Another portion of the right liver and testis samples was excised and placed in trizol for total mRNA quantification of SOCS1, STAT1, and JAK1. Additionally, another portion of each right liver and testis sample was fixed in 10% formalin-saline solution for immunohistochemical analysis of liver and testis PIAS1.

2.4. Liver and testis quantifications of IL-6, TNF-α, iNOS, COX-2, and NF-κB

ELISA assays were performed according to the manufacturer's instructions using pre-coated microplates specific for IL-6, TNF-α, iNOS, COX-2, or NF-κB. The immobilized capture antibody was allowed to bind to the antigens, followed by the addition of a biotinylated goat polyclonal anti-rat detection antibody, resulting in an antibody-antigen-antibody sandwich formation. The addition of avidin-horseradish peroxidase and TMB substrate solution produced a blue color, which is proportional to the concentration of the inflammatory parameter of interest in the sample. The yellow color formed after the addition of the stop solution was read at 450 nm to determine the absorbance.

2.5. Liver and testis mRNA extractions and RT-PCR gene expressions of SOCS1, STAT1, and JAK1

After homogenizing the liver and testis samples, the total mRNA was extracted and purified. Synthesis of cDNA was performed using the extracted and purified total mRNA from the liver and testis samples. The synthesized cDNA was then amplified using PCR to obtain large copies of each gene, using the forward and backward primer sequences (Shine-Gene Corporation, Shanghai, China) listed in Table 2. The amplified genes were subjected to agarose gel electrophoresis, where the band migrations were captured. The intensity of the cDNA of each gene was quantified [46], and the relative mRNA expression of each gene investigated was estimated using β-actin as the housekeeping gene.

Table 2
Sequences (forward and reverse) of genes of interest.

Gene		Sequences
SOCS1	Forward	TAACCCGGTACTCCGTGACT
	Reverse	CTCCCACGTGGTTCCAGAAA
STAT1	Forward	AACCGATGGAGCTCGATGAC
	Reverse	ACTCCTCTGGAGACATGGGAA
JAK1	Forward	GAATGTACTGGGCGTCTTGG
	Reverse	TCAAGGAGTGGGGTTGCTTC
β-Actin	Forward	CCCGCGAGTACAACCTTCTT
	Reverse	CATCGGTAGGTCCGACACAA

2.6. Liver and testis PIAS immunohistochemical assay

Immunohistochemical analyses of the testis and liver were carried out using the method described by Somade et al. [47]. The tissue-mounted slides were allowed to dry overnight, followed by deparaffinization and rehydration using xylene and stepwise concentrations of ethanol (100%, 95%, and 70%). After soaking the slides in 3% hydrogen peroxide, heat-induced antigen retrieval was performed in citrate buffer and cooled in cold water for 5 min. Endogenous peroxidase blocking was carried out for 5 min using 5% BSA. The slides were incubated at room temperature for 60 min with a rabbit polyclonal antibody for PIAS1 (Cloud-Clone Corp, TX, USA) diluted in 1% phosphate buffer saline. Washed slides were then incubated with secondary antibody for 30 min, and visualization of immune complexes was ensured using 0.05% DAB. The slides were counterstained with hematoxylin and viewed under a light microscope to determine the liver and testis-positive stained cells. To avoid any bias, the viewing and scoring of the PIAS1 positive cells were carried out by a pathologist in a neighboring university who was not informed about the entire study.

2.7. Statistical analysis

Results were presented as mean ± SD following data analysis by one-way ANOVA and Tukey test that checked for significant differences among the groups, using Graph Pad Prism version 8.0.2. p-value < 0.05 is statistically significant.

3. Results

3.1. Effects of SACI treatments on liver and testis levels of TNF-α in MCEL-administered rats

The results of liver and testis TNF-α concentrations are presented in Fig. 1A and B, respectively. MCEL administration significantly increased ($p < 0.05$) the hepatic and testicular pro-inflammatory cytokine by 22.06% (Fig. 1A) and 330.80% (Fig. 1B) compared to the control group. Hepatic TNF-α (Fig. 1A) was significantly ($p < 0.05$) decreased by 25 (25.21%), 50 (34.20%), and 75 (41.61%) mg/kg of SACI, while the testicular TNF-α level (Fig. 1B) was significantly lowered ($p < 0.05$) by 30.73%, 28.13%, and 59.98% when rats were treated with 25, 50, and 75 mg/kg of SACI, respectively, compared to the MCEL-only group. TNF-α levels in both tissues following the administration of 75 mg/kg of SACI only were at levels comparable to the control group.

3.2. Effects of SACI treatments on liver and testis levels of IL-6 in MCEL-administered rats

Fig. 2A and B shows the results of hepatic and testicular concentrations of IL-6, respectively. A significant increase ($p < 0.05$) by 11.77% was observed in the concentration of liver IL-6 (Fig. 2A) after MCEL administration compared to the control group. Similarly, a significant increase ($p < 0.05$) by 233.49% was recorded in testicular IL-6 following MCEL administration compared to control (Fig. 2B). Treatment with SACI at 25, 50, and 75 mg/kg body weight significantly decreased ($p < 0.05$) the hepatic IL-6 (Fig. 2A) by 27.74%, 39.86%, and 46.89%, respectively, while the testicular IL-6 concentration (Fig. 2B) was significantly reduced ($p < 0.05$) by 26.56%, 23.89%, and 60.58%, respectively, compared to MCEL-only rats. IL-6 levels in both tissues following the administration of 75 mg/kg of SACI only were at levels comparable to the control group.

3.3. Effects of SACI treatments on liver and testis levels of iNOS in MCEL-administered rats

Fig. 3A and B shows the results of liver and testis iNOS concentrations, respectively. Liver (Fig. 3A) and testis (Fig. 3B) concentrations of

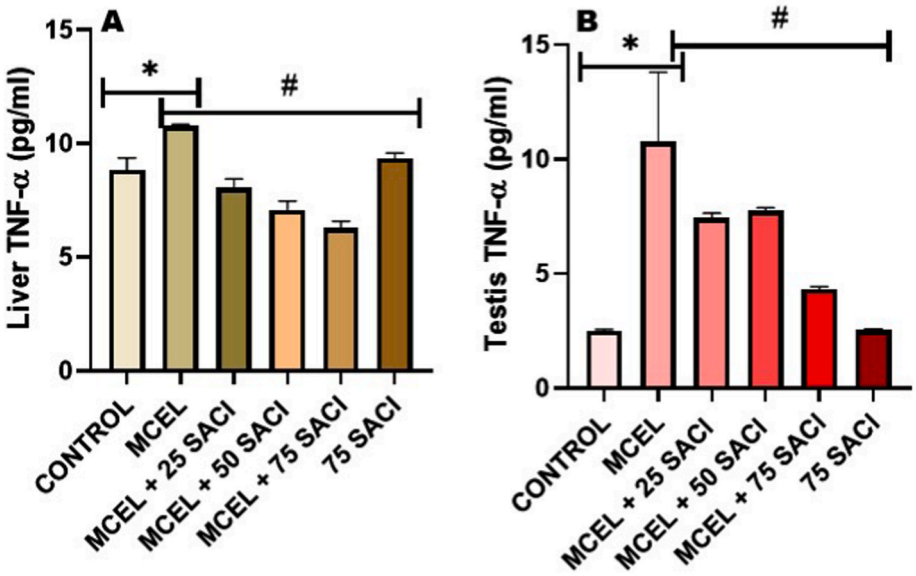

Fig. 1. Effect of SACI treatments on hepatic (A) and testicular (B) TNF-α levels in MCEL administered rats. Each bar represents mean ± SD (n = 3). *Significantly different from control ($p < 0.05$). #Significantly different from MCEL ($p < 0.05$). MCEL = methyl cellosolve, 25 SACI = 25 mg/kg syringic acid, 50 SACI = 50 mg/kg syringic acid, 75 SACI = 75 mg/kg syringic acid.

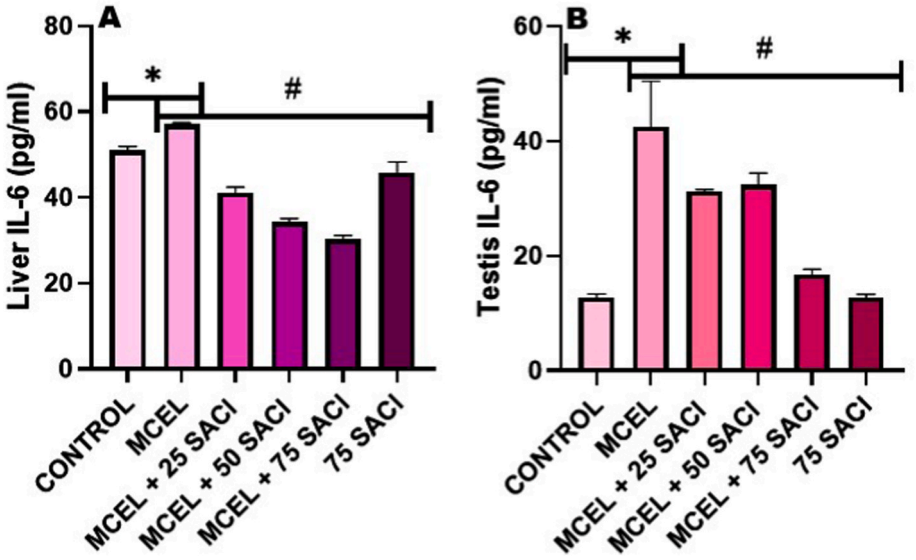

Fig. 2. Effect of SACI treatments on hepatic (A) and testicular (B) IL-6 levels in MCEL administered rats. Each bar represents mean ± SD (n = 3). *Significantly different from control ($p < 0.05$). #Significantly different from MCEL ($p < 0.05$). MCEL = methyl cellosolve, 25 SACI = 25 mg/kg syringic acid, 50 SACI = 50 mg/kg syringic acid, 75 SACI = 75 mg/kg syringic acid.

iNOS were significantly elevated ($p < 0.05$) by 108.86% and 173.44%, respectively, when MCEL only was administered compared to the control. SACI treatments at 50 and 75 mg/kg significantly ($p < 0.05$) reduced the hepatic (Fig. 3A) iNOS concentration by 32.93% and 43.42%, respectively, compared to MCEL only, while all tested doses significantly reduced ($p < 0.05$) the testicular iNOS (Fig. 3B) concentration by 19.92% (25 mg/kg), 34.78% (50 mg/kg), and 49.22% (75 mg/kg) compared to MCEL only. iNOS levels in both tissues following the administration of 75 mg/kg of SACI only were at levels comparable to the control group.

3.4. Effects of SACI treatments on liver and testis levels of COX-2 in MCEL-administered rats

Fig. 4A and B shows the results of COX-2 concentrations in the liver and testis, respectively. The concentrations of the liver (Fig. 4A) and testis (Fig. 4B) COX-2 were significantly increased ($p < 0.05$) by 65.88% and 181.66%, respectively, after exposure to MCEL compared with the control. SACI treatments at 25, 50, and 75 mg/kg significantly increased ($p < 0.05$) the liver (Fig. 4A) COX-2 concentration by 27.95%, 15.53%, and 9.67%, respectively, and the testis (Fig. 4B) COX-2 concentration by 20.83%, 26.84%, and 63.80%, respectively, compared with MCEL only. The COX-2 level in the liver of rats was significantly elevated ($p < 0.05$) by SACI at 75 mg/kg only compared with the control, while the testis COX-2 level was at a level comparable with control after the

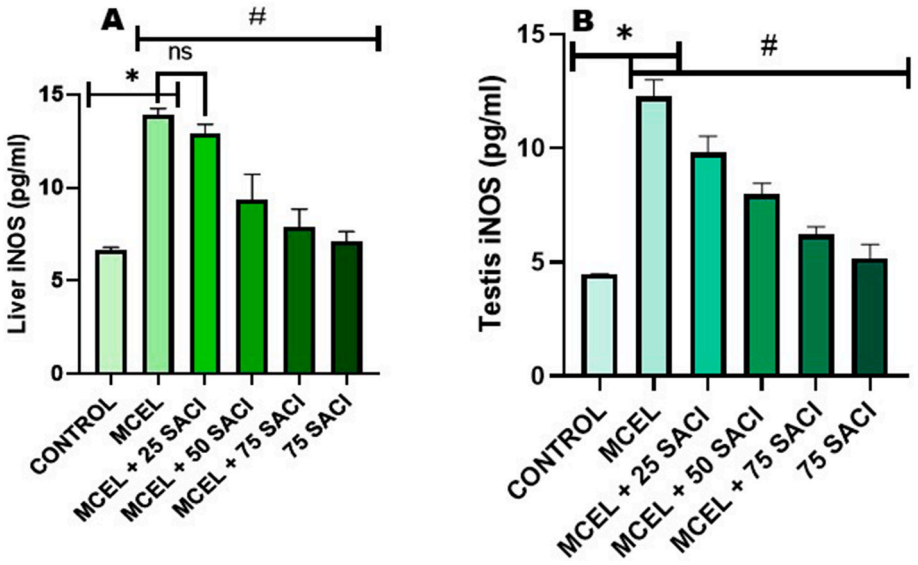

Fig. 3. Effect of SACI treatments on hepatic (A) and testicular (B) iNOS levels in MCEL administered rats. Each bar represents mean ± SD (n = 3). *Significantly different from control ($p < 0.05$). #Significantly different from MCEL ($p < 0.05$). MCEL = methyl cellosolve, 25 SACI = 25 mg/kg syringic acid, 50 SACI = 50 mg/kg syringic acid, 75 SACI = 75 mg/kg syringic acid.

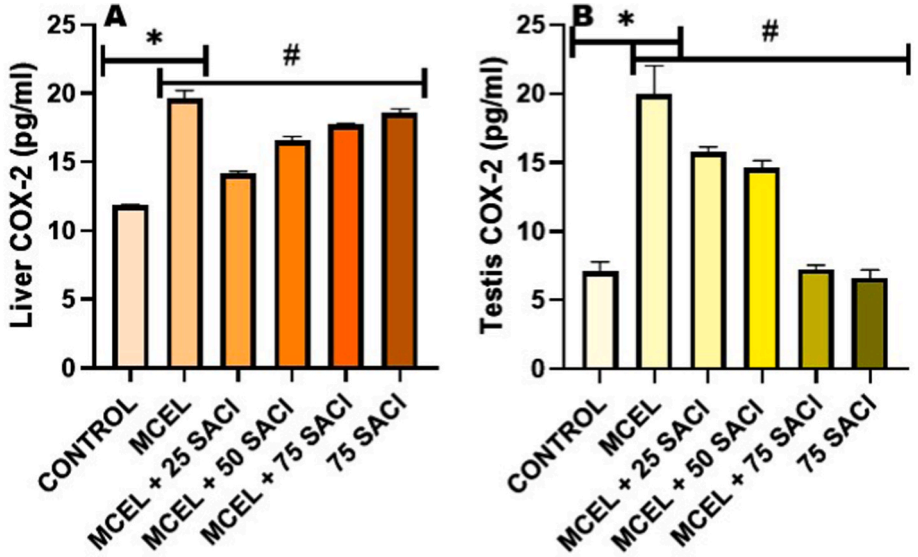

Fig. 4. Effect of SACI treatments on hepatic (A) and testicular (B) COX-2 levels in MCEL administered rats. Each bar represents mean ± SD (n = 3). *Significantly different from control ($p < 0.05$). #Significantly different from MCEL ($p < 0.05$). MCEL = methyl cellosolve, 25 SACI = 25 mg/kg syringic acid, 50 SACI = 50 mg/kg syringic acid, 75 SACI = 75 mg/kg syringic acid.

administration of 75 mg/kg of SACI only.

3.5. Effects of SACI treatments on liver and testis levels of NF-κB in MCEL-administered rats

Results of hepatic and testicular concentrations of NF-κB were presented in Fig. 5A and B respectively. Liver (Fig. 5A) and testis (Fig. 5B) NF-κB concentrations were significantly increased ($p < 0.05$) following MCEL administrations by 73.75% and 74.01% respectively, compared with control. Significant reductions ($p < 0.05$) in liver (Fig. 5A) concentration of NF-κB by 30.12%, 15.08%, and 10.55% were recorded after SACI treatments at 25, 50, and 75 mg/kg respectively, while significant reductions ($p < 0.05$) in testis (Fig. 5B) concentration of NF-κB by 19.57%, 14.01%, and 50.07% were recorded after SACI treatments at 25, 50, and 75 mg/kg respectively, compared with MCEL only. NF-κB level in the liver of rats was also elevated significantly ($p < 0.05$) by SACI at 75 mg/kg only compared with control, while testis NF-κB level is at a level comparable with control after the administrations of 75 mg/kg of SACI only.

3.6. Effects of SACI treatments on liver and testis total mRNA expressions of JAK1 in MCEL-administered rats

Fig. 6A and B shows the results of hepatic and testicular total mRNA expressions of JAK1, respectively. The hepatic (Fig. 6A) total mRNA level of JAK1 was significantly increased ($p < 0.05$) by 138.96% when MCEL was administered compared with the control. SACI treatments significantly decreased ($p < 0.05$) the liver (Fig. 6A) expression of JAK1 mRNA by 1.65% (25 mg/kg), 38.59% (50 mg/kg), and 44.57% (75 mg/kg) compared with MCEL only. In the testis (Fig. 6B), MCEL

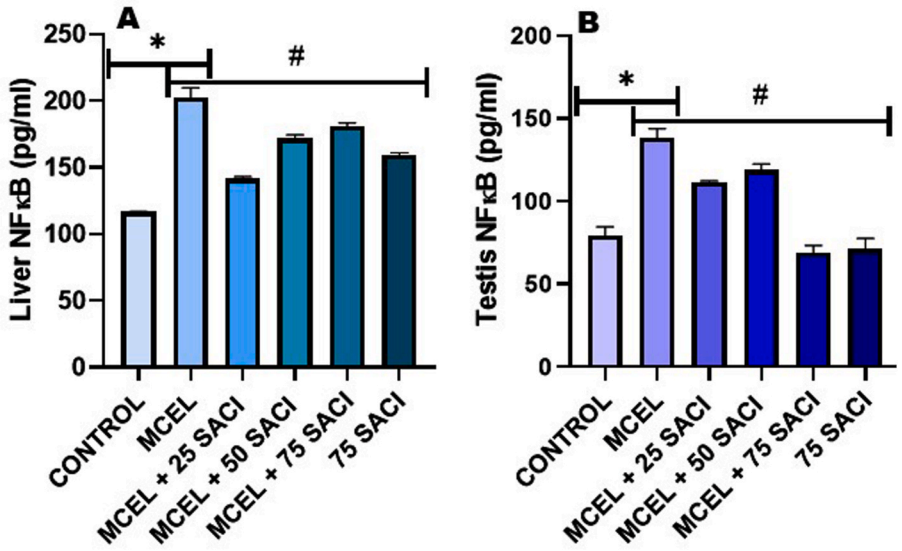

Fig. 5. Effect of SACI treatments on hepatic (A) and testicular (B) NF-κB levels in MCEL administered rats. Each bar represents mean ± SD (n = 3). *Significantly different from control ($p < 0.05$). #Significantly different from MCEL ($p < 0.05$). MCEL = methyl cellosolve, 25 SACI = 25 mg/kg syringic acid, 50 SACI = 50 mg/kg syringic acid, 75 SACI = 75 mg/kg syringic acid.

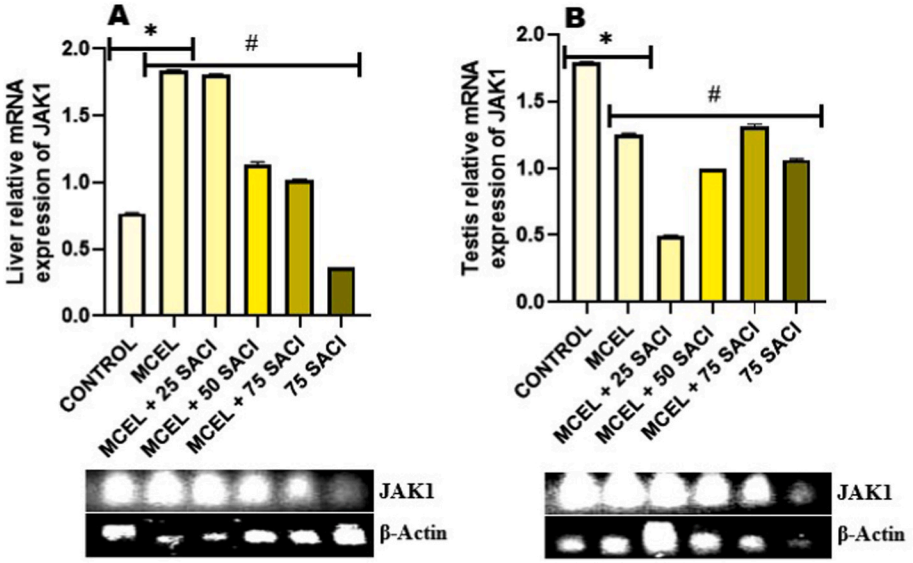

Fig. 6. Effect of SACI treatments on hepatic (A) and testicular (B) JAK1 mRNA levels in MCEL administered rats. Each bar represents mean ± SD (n = 3). *Significantly different from control ($p < 0.05$). #Significantly different from MCEL ($p < 0.05$). MCEL = methyl cellosolve, 25 SACI = 25 mg/kg syringic acid, 50 SACI = 50 mg/kg syringic acid, 75 SACI = 75 mg/kg syringic acid.

administrations significantly reduced ($p < 0.05$) the mRNA level of JAK1 by 30.17% compared with the control. Further significant reductions ($p < 0.05$) by 60.00% and 16.64% were seen after SACI treatments at 25 and 50 mg/kg, respectively, and a significant increase ($p < 0.05$) by 4.80% after SACI treatments at 75 mg/kg, compared with MCEL only. SACI at 75 mg/kg significantly ($p < 0.05$) lowered the hepatic and testicular JAK1 gene expressions compared with the control.

3.7. Effects of SACI treatments on liver and testis total mRNA expressions of STAT1 in MCEL-administered rats

Fig. 7A and B represent the results of liver and testis STAT1 total mRNA expressions respectively. MCEL administrations significantly ($p < 0.05$) increased the hepatic (Fig. 7A) and testicular (Fig. 7B) STAT1 mRNA expressions by 747.37% and 90.79% respectively compared with control. Treatments with SACI at 25 and 50 mg/kg significantly lowered ($p < 0.05$) the liver (Fig. 7A) STAT1 by 20.50% and 56.52% respectively, and testicular (Fig. 7B) STAT1 by 71.03% and 50.34% respectively, compared with MCEL only. In both the liver and testis, 75 mg/kg of SACI significantly increased ($p < 0.05$) STAT1 mRNA by 21.11% (Figs. 7A) and 37.93% (Fig. 7B) compared with MCEL only. SACI at 75 mg/kg only significantly ($p < 0.05$) increased the hepatic and testicular STAT1 mRNA expressions compared with the control.

3.8. Effects of SACI treatments on liver and testis total mRNA expressions of SOCS1 in MCEL-administered rats

Fig. 8A and B depict the results of total mRNA expressions of SOCS1 in the liver and testis, respectively. The SOCS1 mRNA level in the liver (Fig. 8A) significantly increased ($p < 0.05$) by 276.92%, and the SOCS1

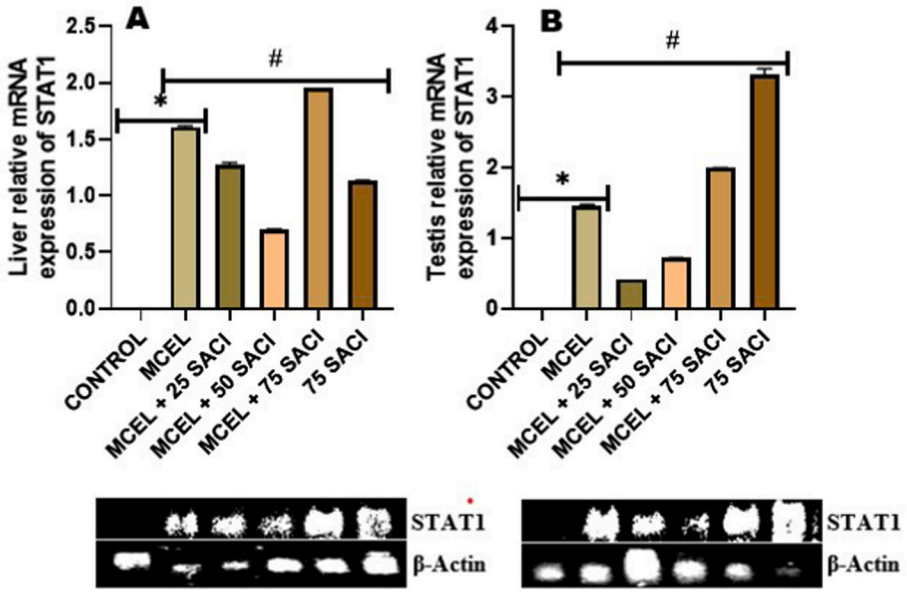

Fig. 7. Effect of SACI treatments on hepatic (A) and testicular (B) STAT1 mRNA levels in MCEL administered rats. Each bar represents mean ± SD (n = 3). *Significantly different from control ($p < 0.05$). #Significantly different from MCEL ($p < 0.05$). MCEL = methyl cellosolve, 25 SACI = 25 mg/kg syringic acid, 50 SACI = 50 mg/kg syringic acid, 75 SACI = 75 mg/kg syringic acid.

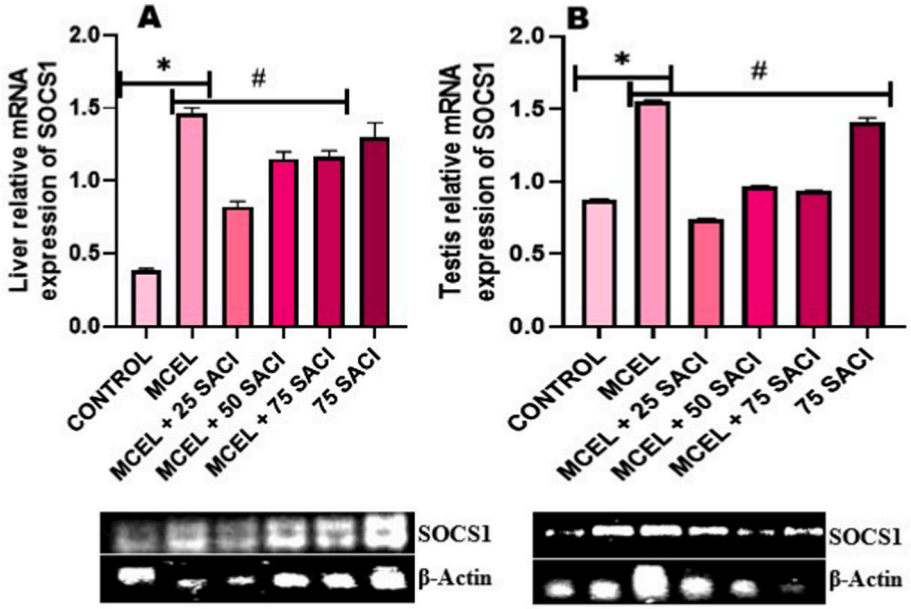

Fig. 8. Effect of SACI treatments on hepatic (A) and testicular (B) SOCS1 mRNA levels in MCEL administered rats. Each bar represents mean ± SD (n = 3). *Significantly different from control ($p < 0.05$). #Significantly different from MCEL ($p < 0.05$). MCEL = methyl cellosolve, 25 SACI = 25 mg/kg syringic acid, 50 SACI = 50 mg/kg syringic acid, 75 SACI = 75 mg/kg syringic acid.

mRNA level in the testis (Fig. 8B) increased by 79.31% after MCEL administrations compared to the control. Treatments with SACI at 25, 50, and 75 mg/kg significantly lowered ($p < 0.05$) the liver (Fig. 8A) SOCS1 mRNA level by 44.22%, 21.77%, and 20.41%, respectively, and testis (Fig. 8B) SOCS1 by 52.56%, 37.82%, and 39.74%, respectively, compared to MCEL only. Like STAT1, only SACI at 75 mg/kg significantly ($p < 0.05$) increased the hepatic and testicular SOCS1 gene expressions compared to the control.

3.9. Effects of SACI treatments on liver and testis total immunohistochemical expressions of PIAS1 in MCEL-administered rats

In the liver (Fig. 9A), administrations of MCEL caused a significant ($p < 0.05$) increase in the protein expression of PIAS1 by 438% compared to its expression in the control rats. SACI treatments at 25 and 50 mg/kg significantly ($p < 0.05$) exacerbated the PIAS1 protein expression by 66.81% and 100.44%, respectively, while treatment at 75 mg/kg significantly ($p < 0.05$) reduced the PIAS1 protein expression by 37.17% compared to rats administered MCEL only (Fig. 9A). Similarly, testis PIAS1 (Fig. 9B) was significantly ($p < 0.05$) elevated by 94.56%

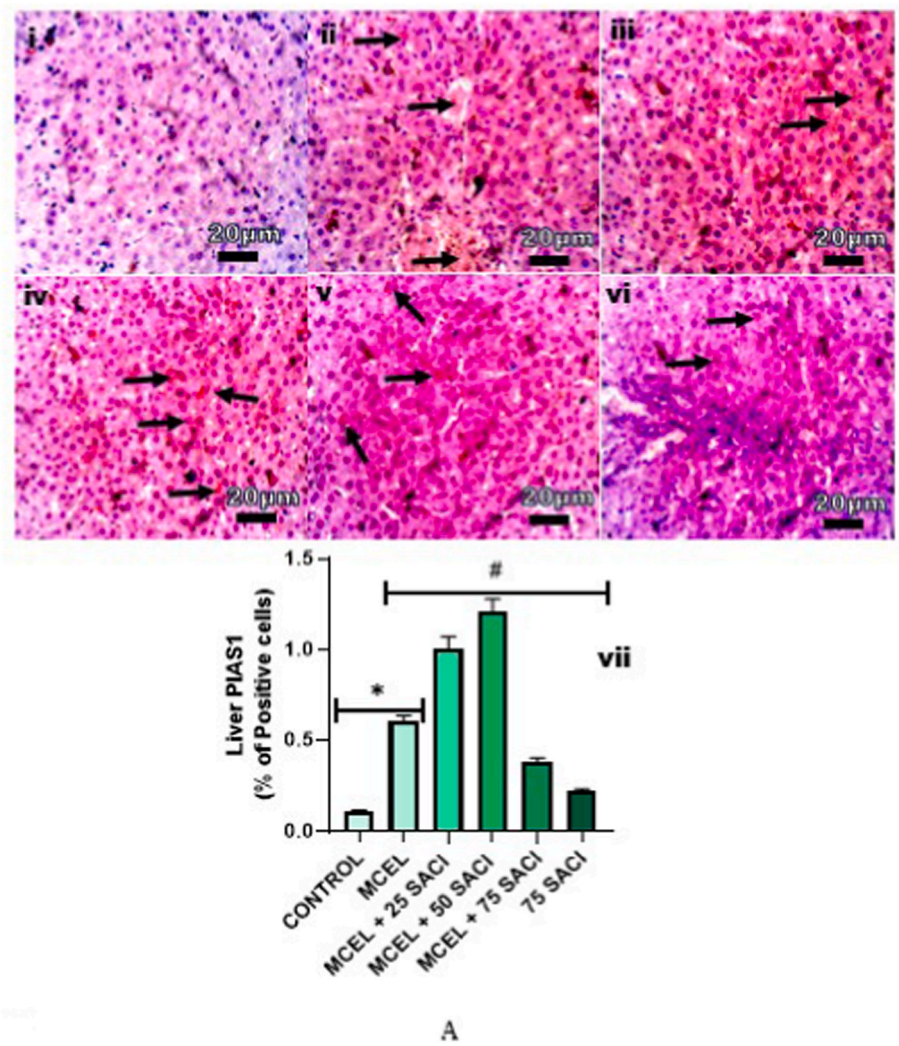

Fig. 9A. Effect of SACI treatments on hepatic PIAS1 immunohistochemical protein expression levels in MCEL administered rats (x400 magnification). i = Control; ii = MCEL only; iii = MCEL + 25 SACI; iv = MCEL + 50 SACI; v = MCEL + 75 SACI; vi = 75 SACI only. Each bar (in Fig. vii) represents mean ± SD (n = 3); *Significantly different from control ($p < 0.05$). #Significantly different from MCEL ($p < 0.05$). MCEL = methyl cellosolve, 25 SACI = 25 mg/kg syringic acid, 50 SACI = 50 mg/kg syringic acid, 75 SACI = 75 mg/kg syringic acid.

compared to the rats in the control group when MCEL was administered for 30 days. Treatments with 25, 50, and 75 mg/kg of SACI significantly ($p < 0.05$) decreased the testicular PIAS1 expression by 26.55%, 90.31%, and 86.25%, respectively, compared to MCEL-only exposed rats (Fig. 9B). The PIAS1 level in the liver following the administrations of 75 mg/kg of SACI was comparable to the control level, while in the testis, it was significantly reduced by 75 mg/kg of SACI only compared to control.

4. Discussion

Cytokines are induced to respond and protect the host cells against injury, irritation, and infection. Examples of these pro-inflammatory cytokines include IL-1β, TNF-α, IL-6, and IFN-γ, which are produced by the same or different cells [33]. Typically, cytokines communicate with neighboring tissues in response to injury or infection. Pro-inflammatory cytokines are secreted by macrophages and play a critical role in the defense against infection, representing a form of adaptive immune response [48]. In our study, MCEL-induced hepato-testicular inflammation was observed, as marked by a significant elevation in the concentrations of IL-6 and TNF-α. The attack on hepatic and testicular cells by MCEL may have caused cellular injuries that resulted in adaptive responses by immune cells and their recruitment to the injured sites, where these pro-inflammatory mediators were released to elicit their pro-inflammatory roles. These results are consistent with previous studies on MCEL by Somade et al. [39,40], where a dose-dependent significant increase in hepatic and testicular levels of TNF-α and IL-6 was observed. SACI at all the doses investigated demonstrated an anti-inflammatory effect, which may be attributed to its antioxidant and cytoprotective properties capable of shielding the tissues against MCEL-induced inflammation. This anti-inflammatory property of SACI has been previously reported against N-nitrosodimethylamine-induced lung fibrogenesis in rats [20].

NF-κB is an inducible transcription factor that regulates several genes associated with processes involved in immunoinflammatory responses [49]. There are 5 structurally related members (p50, p52, p65, c-Rel, and RelB) of this family that mediate the nuclear transcription of target genes by associating with κB enhancer, a specific DNA element [50]. In a basal or unstressed cellular state, NF-κB is found sequestered with its regulator IκB in the cytosol. NF-κB becomes activated following the inducible IκBα degradation that is triggered via phosphorylation by IKK [49]. Upon the separation and degradation of IκB, NF-κB translocates to the nucleus where it drives the transcription of inflammatory genes [51], including pro-inflammatory cytokines, iNOS, COX-2, and so on. From our findings in this study, the significant increase in hepatic and testicular NF-κB after the administrations of MCEL suggests its cytoplasmic activation and involvement in the inflammatory processes in both tissues. The nuclear translocation of the activated NF-κB in the cytosol may have resulted in the nuclear expressions of inflammatory players, including pro-inflammatory cytokines, and this may explain the recorded elevation in the levels of hepatic and testicular TNF-α and IL-6 in this study. This is in line with the work of Natarajan et al. [52], who

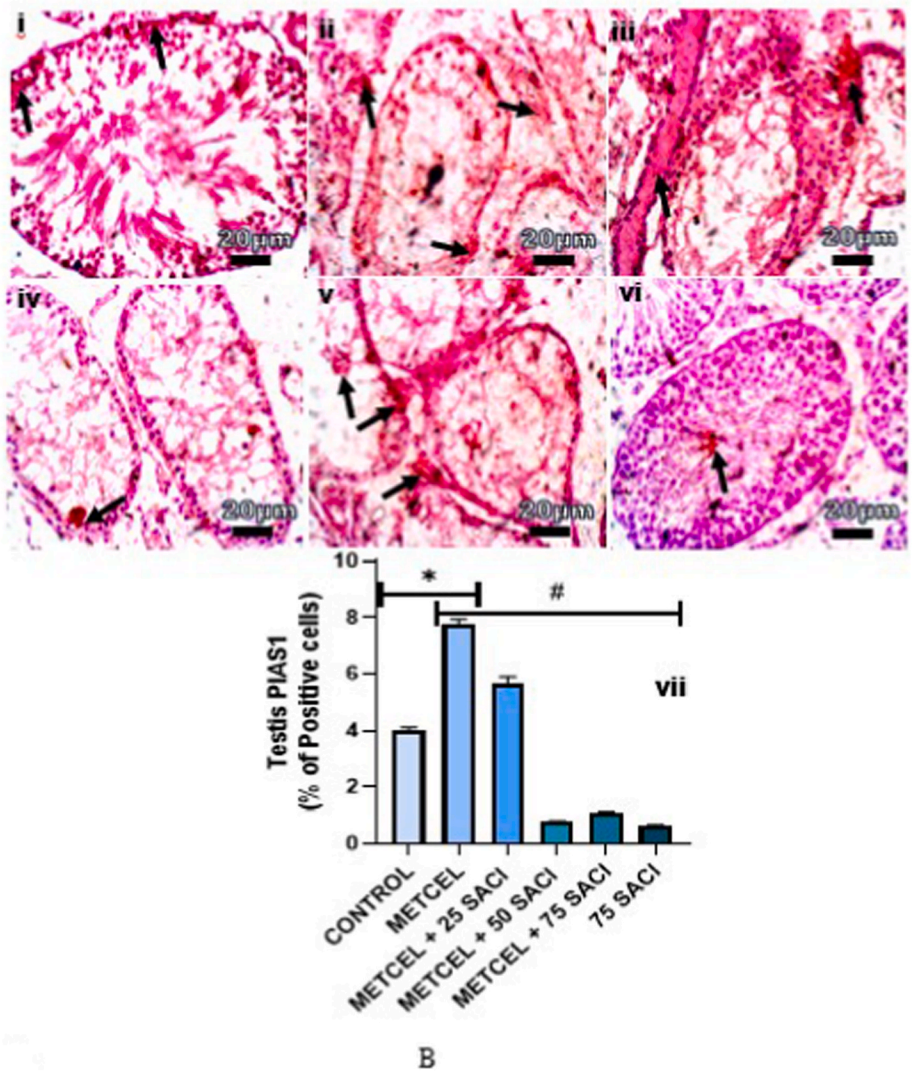

Fig. 9B. Effect of SACI treatments on testicular PIAS1 immunohistochemical protein expression levels in MCEL administered rats (x400 magnification). i = Control; ii = MCEL only; iii = MCEL + 25 SACI; iv = MCEL + 50 SACI; v = MCEL + 75 SACI; vi = 75 SACI only. Each bar (in Fig. vii) represents mean ± SD (n = 3); *Significantly different from control ($p < 0.05$). #Significantly different from MCEL ($p < 0.05$). MCEL = methyl cellosolve, 25 SACI = 25 mg/kg syringic acid, 50 SACI = 50 mg/kg syringic acid, 75 SACI = 75 mg/kg syringic acid.

reported the activation of NF-κB and an increase in mRNA and protein expressions of TNF-α, iNOS, and COX-2 in methotrexate-induced gastrointestinal toxicity in rats. The significant reductions in the testicular and hepatic NF-κB levels at all investigated doses point to the cytoprotective property of SACI, which may be attributed to its rich antioxidant property. SACI may have prevented the cytosolic activation of NF-κB, thereby preventing its ability to exert its nuclear transcription activities. This anti-inflammatory effect of SACI, characterized by the significant decrease in mRNA expressions of NF-κB and TNF-α in isoproterenol-induced cardiotoxicity [53] and thioacetamide-induced hepatic encephalopathy [54] in rats, has also been reported.

Expression of iNOS is mainly controlled by transcription factors [55], which vary depending on the species and cell type. In this study, elevated levels of testicular and hepatic iNOS were observed after MCEL administration, indicating its pro-inflammatory activity. MCEL-induced cellular production of pro-inflammatory cytokines may have caused the cytoplasmic activation of NF-κB, followed by the nuclear transcription of the iNOS genes, as previously demonstrated by methotrexate in the gastrointestinal tract [51] and cyclophosphamide-induced hepatotoxicity [56] in rats. The significant reduction in testicular and hepatic iNOS levels after SACI treatment adds to the evidence of its anti-inflammatory property, which may be due to SACI's ability to reduce the levels of pro-inflammatory cytokines and the cytoplasmic activation of NF-κB, the transcription factor that mediates the nuclear expression of iNOS genes.

The biosynthesis of AA, the precursor for PGs biosynthesis, involves the release of AA from phospholipids by phospholipase A2. PGH2 is subsequently synthesized from AA by COX-1 and COX-2, the two functional cyclooxygenases. COX-2 is an inducible gene and is the major source of PGs, making it a pathological enzyme primarily responsible for tissue inflammation [57]. Thus, the significant increase in liver and testis COX-2 levels observed in this study is an indication of the involvement of MCEL in testicular and hepatic inflammation. Like iNOS, the expression of the COX-2 gene is also controlled by NF-κB. Therefore, MCEL-induced cellular activation of NF-κB may also be responsible for the significant rise in COX-2 levels in both tissues, as previously reported by Noor et al. [58] in cadmium-induced liver damage in rats. The anti-inflammatory action of SACI was demonstrated by the significant decrease in testicular and hepatic COX-2 levels, which can be attributed to the inhibition of NF-κB activation, as discussed earlier. This finding is consistent with the work of Pei et al. [59], who reported the anti-inflammatory action of SACI in gastric cancer cells by reducing the expressions of IL-6, NF-κB, TNF-α, and COX-2. In the liver of rats, the effect of SACI treatments on MCEL-induced elevation in COX-2 protein is dose-dependent, while the opposite is observed in the testis. This may suggest the COX-2-inducing effect of SACI in the liver of rats, as evidenced by the result of liver COX-2 in the group that received 75 mg/kg of SACI only. This COX-2-inducing effect may be due to the rich availability of its substrate, arachidonic acid, which is an unsaturated fatty acid mainly biosynthesized in the liver compared to the testis.

In initiating the canonical JAK-STAT signaling pathway, type 1 or 2 cytokines bind to their relevant receptors. These receptors, whether type 1 or 2, are structurally distinct and oligomerized after a cytokine binds to them. After oligomerization, the intracellular cytokine receptor subunits separate, moving the JAKs associated with that receptor apart and relieving the constitutive inhibition, leading to their activation [60]. This results in the autophosphorylation of JAKs, which then phosphorylate the receptors at the tail or intracellular portion, acting as STAT docking sites [61]. Upon binding of STATs (a nuclear transcription factor), JAKs phosphorylate them, and the inactive STAT monomers form active homodimers that translocate into the nucleus, initiating the transcription of genes [62]. In this study, a significant increase in the total mRNA expressions of hepatic JAK1 and STAT1, and a significant decrease and increase in testicular JAK1 and STAT1 respectively were recorded in rats administered only MCEL. This may be due to the significant production of pro-inflammatory cytokines (e.g., IL-6, TNF-α) that was recorded, which may have bound to their corresponding receptors and initiated the JAK-STAT signaling. This may have also caused the significant increase in the expressions of inflammatory mediators (COX-2 and iNOS) recorded in this study, which agrees with the study of Nunes et al. [63], who reported the activation of the JAK-STAT pathway and increased expressions of COX-2 and iNOS in cytokine-stimulated colon epithelial cells. Additionally, the JAK-STAT signaling may have been activated to induce the genes needed to prevent apoptosis and ensure cell survival [64]. SACI treatments significantly reduced the mRNA expressions of JAK1 and STAT1 in the liver and testis of MCEL-challenged rats, suggesting the anti-inflammatory role of SACI. The ability of SACI to lower the levels of all the inflammatory markers in this study may be the mechanism by which it inhibits the JAK-STAT signaling pathway. Furthermore, we observed that the STAT1 inhibitory effect of SACI is best demonstrated at lower doses (25 and 50 mg/kg) and that SACI at high doses (75 mg/kg) may have a STAT1-activation-promoting effect, as judged by the results of liver and testis STAT1 gene expressions in the two groups of rats administered 75 mg/kg of SACI.

SOCS proteins play an advantageous role in regulating the JAK-STAT signaling by terminating the pathway. Therefore, a deficiency of SOCS or a lowered expression of SOCS may cause changes in the responses of cytokines [65]. In an unstimulated state, SOCS protein members are not highly expressed, but they are promptly induced and transcriptionally expressed at elevated levels upon cytokine stimulation and activation of the JAK-STAT pathway [66]. In addition to its anti-inflammatory role, SOCS1 is also a tumor suppressor [67], so the downregulation of SOCS1 can aid human cancer progression. In this study, upregulation of total mRNA expressions of SOCS1 in the testis and liver was recorded in response to MCEL induction, which further confirms the MCEL-induced inflammation in these organs of rats. The tissue inflammation induced by MCEL, resulting in a significant increase in the production of pro-inflammatory cytokines and eventual activation of the JAK-STAT signaling pathway, may be responsible for the increased SOCS1 mRNA expressions. Moreover, the MCEL-induced increase in SOCS1 mRNA expressions in the liver and testis of rats may be a mode of adaptive response to the activated JAK-STAT pathway, which is needed to put the signaling pathway in check. Our findings also revealed a significant reduction in the liver and testis SOCS1 mRNA expressions following SACI treatments, which confirms SACI's anti-inflammatory effect. The reduced liver and testis SOCS1 expressions can be attributed to SACI's ability to lower the production of pro-inflammatory cytokines and inhibit MCEL-induced JAK and STAT mRNA expressions that were recorded in this study.

La Manna et al. [68] reviewed the efficacy of KIRESS in treating tumor-bearing mice by targeting disseminated tumor cells in the primary tumor, bone marrow, and spleen. Treatment with KIRESS resulted in reduced tumor growth and complete elimination of pulmonary metastasis in mice [68]. The drug's mechanism of action was specific to the SOCS3-pSTAT3-NF-kB pathway, reducing the phosphorylation of STAT3 and NF-kB (p65), as well as the expression of inflammatory cytokines, similar to the whole SOCS3 protein [69]. PS5, a peptidomimetic of KIR-SOCS1, has been shown to reduce inflammation and oxidative stress in atherosclerosis by decreasing the expression of NOX1 and NOX4 genes, upregulating antioxidant genes, and reducing the size of atheroma plaque, lipid content, and accumulation of immune cells [70]. Another study by La Manna et al. [71] suggests that peptidomimetics of SOCS3 could be a potential therapeutic option for diseases characterized by activated inflammatory cytokines. They found that aromatic contribution plays a significant role in the recognition of JAK2. In a related study, the internal cyclic PS5 analogs demonstrated the ability to inhibit JAK-mediated tyrosine phosphorylation of STAT1, reduce cytokine-induced proinflammatory gene expression, oxidative stress generation, and cell migration [72]. The study contributed to the development of low-molecular-weight proteomimetics with long-term cellular effects and added to the process of converting bioactive peptides into drugs. Similarly, La Manna et al. [73] used a combination of techniques to investigate the impact of cycle size and unnatural building blocks on the interaction of compounds with JAK2. They found that the cycle size is important for the interaction with JAK2 and that the substitution of native residues with un-natural building blocks can maintain low-micromolar affinity and increase serum stability, enhancing their understanding of the structure required for SOCS1/JAK2 recognition and helping in developing drug-like compounds.

The PIAS protein family, consisting of PIAS1, PIAS2, PIAS3, and PIAS4, is involved in regulating transcription [74]. PIAS, as suggested by its name, is a STAT inhibitor. PIAS can only interact with dimerized STAT following phosphorylation by JAK (a kinase). Interaction with STAT monomers does not occur [74]. PIAS primarily controls signal transduction by inhibiting the DNA-binding ability of transcription factors [75], enhancing the sumoylation of transcription factors, recruiting other co-regulatory agents [76], and binding to transcription factors to control transcription [77]. In this study, similar effects of MCEL on the protein expression of PIAS1 were observed in the liver and testis of rats that were administered only MCEL. The significantly elevated PIAS1 protein expression in both tissues suggested the induction and adaptive response of PIAS1 to the activated and dimerized STAT1. This increase in PIAS1 protein expression in the two compartments following MCEL-only administration compared to control may be attributed to a response to the recorded activation of the JAK/STAT signaling pathway in this study. This way, there could be a blockage of the activated and dimerized STAT from translocating into the nucleus, where it can bind to the promoter of genes that can exacerbate inflammation in the liver and testis. In both the liver (at 75 mg/kg only) and testis (at all doses), SACI demonstrated STAT inhibitory and regulatory effects, as evidenced by the significant reduction in activated PIAS1 protein expression in rats.

5. Conclusion

NF-κB is a transcription factor that plays a role in the nuclear transcription of pro-inflammatory genes. On the other hand, the JAK-STAT signaling pathway is activated following the binding of pro-inflammatory cytokines to their receptors. Therefore, there is a link between these two signaling pathways, as the activation of one pathway is required to initiate and activate the other. We propose that this may be the mechanism by which MCEL causes tissue inflammation in rats. SACI, on the other hand, demonstrated a potent anti-inflammatory effect against MCEL-induced inflammation by downregulating the hepatic and testicular NF-κB-iNOS-COX-2 and JAK-STAT signaling in rats. The impressive performance of SACI in this regard suggests its potential use in the treatment of inflammatory-associated diseases.

Limitations

The unavailability of funds impacted the experimental design,

particularly the sample size, as we were limited to only 6 groups of 5 rats. This limitation forced us to drop several inflammatory markers and other signaling pathways linked with inflammation that we originally intended to investigate. Additionally, due to the high exchange rate of the US dollar to the Nigerian naira, the prices of kits and chemicals were prohibitively expensive, which prevented us from analyzing all samples in each group.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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