



# Syringic and ascorbic acids prevent NDMA-induced pulmonary fibrogenesis, inflammation, apoptosis, and oxidative stress through the regulation of PI3K-Akt/PKB-mTOR-PTEN signaling pathway

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## ARTICLE INFO

### Keywords:

Syringic acid  
Ascorbic acid  
N-Nitrosodimethylamine  
Fibrosis  
Signaling  
Lung

## ABSTRACT

Idiopathic lung fibrosis (ILF) is a severe and life threatening lung disorder that is characterized by scarring of lung tissue, leading to thickening and stiffening of affected areas. This study looked at the role played by PI3K-Akt/PKB-mTOR signaling pathway in the pathogenesis of N-Nitrosodimethylamine (NDMA)-induced lung fibrotic injury, and the effects of syringic acid (SYR) and ascorbic acid (ASC) treatments in male Wistar rats. Pulmonary fibrosis was induced by intraperitoneal injection of 10 mg/kg NDMA once daily, thrice (consecutively) a week for four weeks, and this condition was treated daily with SYR (50 mg/kg) and ASC (100 mg/kg) acids orally for four weeks. Fibrogenesis, following NDMA administration was marked by a significant increase in collagen-1 and  $\alpha$ -SMA levels, while oxidative stress was marked by a significant decrease in GSH level, GST, GPx, CAT, and SOD activities. Also, NDMA significantly increased lung Bax, p53, caspase-3, TNF- $\alpha$ , IL-1 $\beta$ , NFkB, and decreased Bcl-2, mdm2, cyclin D1 and Nrf-2 levels. Looking at the PI3K-Akt-mTOR signaling pathway, NDMA administration significantly activated lung PI3K, Akt, and mTOR, and deactivated PTEN, FoxO1 and TSC2. Treatments with SYR and ASC significantly reduced oxidative stress by restoring the antioxidant systems via Nrf2 activation, decreased the levels of inflammatory markers through inhibition of NFkB, downregulated p53, Bax, and caspase-3 via up-regulation of mdm2 and cyclin D1. SYR and ASC also regulated the PI3K-Akt-mTOR signaling pathway via the deactivation of PI3K, Akt, and mTOR, and up-regulation of PTEN, FoxO1 and TSC2. Overall, SYR and ASC modulate the PI3K-Akt-mTOR signaling pathway via inhibition of oxidative stress, inflammation and apoptosis in NDMA-induced lung fibrosis.

## 1. Introduction

N-Nitrosodimethylamine (NDMA) is a genotoxic chemical widely seen in the environment in nitrate or nitrite exposed foods, some beverages, and tobacco smoke. Reported tissues where NDMA is capable of inducing cancer are kidney, lung, and liver [1–3]. NDMA is an extremely lethal toxin that is metabolized by the liver microsomal cytochrome P450 (2E1) [4,5], and it is a known inducer of tissue fibrosis [6].

Fibrosis, a disorder marked by the scarring and thickening of connective tissue due to aggregation of extracellular matrix proteins, is commonly related with chronic tissue inflammation [7]. Chronic state of this disease usually results in widespread disorganization of normal

tissue architecture, accounting for about 45% of mortality in the developed world [8]. Fibrosis can result in virtually all tissues, but idiopathic lung fibrosis (ILF) [9] is a more common fibrotic disease of major concern. ILF, the commonest type of interstitial pulmonary disease is marked by a chronic, progressive and irreversible course having a median survival of 3–5 years [10,11]. Presently, no potent treatment for lung fibrosis has been found, other than pulmonary transplantation. Immediate pathological signs are alveolar epithelial cell injury, persistent inflammatory cell infiltration and diffuse fibrotic alveolitis [12]. Subsequently, fibroblasts are activated, which metamorphose to a myofibroblastic character [12]. These myofibroblastic cells lead to the extracellular matrix and collagen accumulation in the lungs, whereby normal lung tissues are replaced by fibrotic scarring that are

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<https://doi.org/10.1016/j.metop.2022.100179>

Received 8 February 2022; Received in revised form 12 March 2022; Accepted 13 March 2022

Available online 17 March 2022

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**Terminologies and abbreviations**

NO	nitric oxide	$\alpha$ -SMA	alpha smooth muscle actin
GSH	reduced glutathione	PI3K	phosphoinositide 3-kinase
MDA	malondialdehyde	Akt/PKB	protein kinase B
CAT	catalase	mTOR	mechanistic target of rapamycin
GST	glutathione S-transferase	PTEN	phosphatase and tensin homolog
SOD	superoxide dismutase	FoxO1	forkhead box protein O1
GPx	glutathione peroxidase	K-ras	Kirsten rat sarcoma viral oncogene
TNF- $\alpha$	tumor necrosis factor alpha	S6K	ribosomal protein S6 kinase beta-1
IL-1 $\beta$	interleukin 1 $\beta$	VEGF- $\alpha$	vascular endothelial growth factor alpha
Bcl-2	B-cell lymphoma 2	IRS-1	insulin receptor substrate 1
Bax	Bcl-2 associated X	NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
p53	tumor suppressor protein	PDPK1	3-phosphoinositide dependent protein kinase 1
mdm2	mouse double minute 2	TSC2	tuberous sclerosis complex 2
Nrf2	nuclear factor erythroid 2-related factor 2	Rheb	Ras homolog enriched in brain

honeycomb-like in nature [13]. Fibrosis is initiated by lung inflammation, and inability to repair epithelial cell injury is a determining factor in the pathogenesis of fibrosis [12]. Activation of epithelial cells result in the production of cytokines, growth factors, fibroblast growth factor and tumor necrosis factor that initiate fibroblasts migration and proliferation [14]. In failed epithelial repair, there is inability of the damaged tissue to heal and properly resolve, leading to a continuous fibroproliferative state [14]. Fibrosis can be reversed if the underlying cause is abolished. Hence, agents that have anti-fibrotic properties could be utilized to manage and treat these conditions [15].

Phenolic compounds possess aromatic ring-containing hydroxyl groups and are rich sources of antioxidants. Of these phenolic compounds, hydroxybenzoic and hydroxycinnamic acids are two major kinds located in plant parts. Syringic acid (SYR), a phenolic acid derivative of hydroxybenzoic acid is found abundantly in olives and grapes, as well as leaves of *Alpinia calcarata* Roscoe [16]. SYR has reactive species scavenging ability and has been reported to have strong efficacy in the treatment of microbial infection, cancer, inflammation, diabetes, cardiovascular diseases, and neuronal damage [17,18]. Methoxy groups in the structure of SYR confer its therapeutic properties. SYR can mop free radicals, and can coordinate the activities of certain enzymes and numerous transcription factors that have roles in the etiology of cancer, inflammation, angiogenesis and diabetes [17]. In addition, SYR possesses neuro-protective activities against oxidative stress-induced axonal degeneration in sciatic nerves following ischemic injury in rodents [19]. In past studies, SYR improved liver [20] and kidney (diabetic neuropathy) complications in rats [21].

Ascorbic acid (ASC) is a water soluble vitamin, which has ability to quench free radicals, regenerate vitamin E, involved in the biosynthesis of collagen where it serves as a cofactor for prolyl and lysyl hydroxylases, and regulation of gene expression [22]. Intake of ASC lowers the production of pro-inflammatory cytokines, and in connection to cardiovascular diseases, ASC blocks the oxidation of low density lipoprotein, halts lipid peroxidation, and raises the levels of glutathione [23–25]. Antioxidant property of ASC confers its ability to suppress the production of beta-amyloid peptide and glutamate-mediated excitotoxicity [26].

George et al. [6] have reported that rat model of NDMA-induced fibrosis is a good and reproducible model. Studies focusing on treatments of lung fibrosis have not been well reported, which necessitated the need for this study. Therefore, in this study, we investigated the involvement of the PI3K-Akt/PKB-mTOR signaling pathway in NDMA-induced lung fibrotic injury, and the protective efficacy and mechanisms of action of SYR and ascorbic acid (ASC) in male Wistar rats.

**2. Materials and methods****2.1. Test materials, kits, and chemicals**

N-nitrosodimethylamine (NDMA; C<sub>2</sub>H<sub>6</sub>N<sub>2</sub>O) is manufactured by Sigma Chemical Co., Saint Louis, MO, USA, with CAS number 62-75-9, and percentage purity of 98%. Syringic acid (SYR; C<sub>9</sub>H<sub>10</sub>O<sub>5</sub>) with CAS number 530-57-4 and percentage purity of 98% is a product of AK Scientific, 30023 Ahem Ave, Union City CA, 94587 USA. Ascomed ascorbic acid tablets are product of Kunimed Pharmachem Ltd, 18/20, Mortune Avenue, Valley Estate, Ikeja, Lagos, Nigeria. Enzyme linked immunosorbent assay (ELISA) kits for the estimations of pulmonary levels of pro-inflammatory cytokines (TNF- $\alpha$  and IL-1 $\beta$ ), and apoptotic parameters (caspase-3, Bcl-2, and Bax) were purchased from Cusabio Technology Llc, Houston, TX, USA. Primary monoclonal antibodies for immunohistochemical detection of collagen type 1, mdm-2, p53, cyclin D1, Nrf2, and  $\alpha$ -SMA were gotten from Santa Cruz Biotechnology Inc, Dallas, Texas 75220, USA. Primers (Oligonucleotide sequence) for K-ras, PI3K, PDPK1, Akt, NFkB, mTOR, S6K, IRS-1, VEGF- $\alpha$ , FoxO1, PTEN, and TSC2 for the reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were purchased from Shanghai ShineGene Molecular Bio-Technologies, Inc., Wuhe Road, Minhang District, Shanghai, 201109, China. Trizol for ribonucleic acid extraction was purchased from Solarbio Life Science and Co. Ltd, Beijing China. Other chemicals and reagents were purchased from well-known and reputable sources of international recognition.

**2.2. Experimental animals**

Healthy male albino rats (n = 30; average weight of 200 g) of Wistar strain, were obtained from an experimental animal breeding house located near the university. The rats were kept in experimental cages in the animal housing facility of the Department of Biochemistry, and they all have unrestricted access to water and food.

**2.3. Experimental design**

Approval was granted by the Departmental Committee in-charge of the handling and use of experimental animals, before the commencement of this study. Following two (2) weeks of acclimatization of the rats to their new environment, they were separated into six (6) groups of five (5) rats each, based on their body weights and were administered test substances as shown in Table 1.

**Table 1**

Grouping and administration of test substances.

Group	Substance administered	Duration
Group 1	1 ml/kg of saline	28 days
Group 2	10 mg/kg of 1% w/v of NDMA dissolved in saline	3 consecutive days a week for 4 weeks [27,28].
Group 3	10 mg/kg of 1% w/v of NDMA +50 mg/kg SYR [29,30] dissolved in distilled water	3 consecutive days a week for 4 weeks (NDMA) and 28 days (SYR)
Group 4	10 mg/kg of 1% w/v of NDMA + 100 mg/kg ASC [31] dissolved in distilled water	3 consecutive days a week for 4 weeks (NDMA) and 28 days (ASC)
Group 5	50 mg/kg SYR only	28 days
Group 6	100 mg/kg ASC only	28 days

## 2.4. Sacrifice and sample collections

Rats were sacrificed 24 h after the last administrations by cervical dislocation. Rats were humanely handled by abiding with the written guidelines available for the use and handling of research animals [32]. Lung of each rat was harvested, washed in cold 0.9% NaCl, dried on clean filter paper, and their weight was recorded. From the samples, a portion was cut and homogenized in phosphate buffer (pH of 7.4; concentration of 0.1 M). The lung homogenate was separated using a speed of 5000 rpm for duration of 10 min, and the resulting supernatant was used for the estimation of interested biochemical parameters.

## 2.5. Determination of lung MDA, NO and GSH levels

Concentration of lung MDA was carried out by following the method of Buege and Aust [33]. Lung NO concentration was estimated by using Griess reagent (that detects nitrite ion), according to the method of Green et al. [34] while lung GSH concentration was assayed for by following the method of Moron et al. [35].

## 2.6. Estimation of lung GST, GPx, SOD, and CAT activities

Assay for the estimation of GST activity was carried out using the protocol of Habig et al. [36] based on enzyme-catalyzed condensation of glutathione with the model substrate, 1-chloro-2,4-dinitrobenzene. For GPx activity, the protocol of Rotruck et al. [37] was used, SOD assay was done following the protocol of Misra and Fridovich [38], while CAT activity was checked using the protocol of Sinha [39].

## 2.7. Quantification of the levels of apoptotic (caspase-3, bax and bcl-2) and pro-inflammatory

(TNF- $\alpha$  and IL-6) parameters

For the quantification of lung Bcl-2, caspase-3, Bax, TNF- $\alpha$  and IL-1 $\beta$  levels, ELISA method was used as described in the protocol inserted in their respective kit (Cusabio ELISA kits), reported by Somade et al. [40].

## 2.8. Immunohistochemistry of lung collagen type 1, $\alpha$ -SMA, Nrf-2, mdm-2, p53 and cyclin D1

Lung protein expressions of these parameters (based on antigen-antibody association) were investigated by following the protocols described by Somade et al. [41]. The percentage of tissue-stained positive cells was scored. Expressions of these proteins were then quantified using Fiji Image J software.

## 2.9. Reverse transcriptase (RT) gene expressions of lung ras, PI3K, PDPK1, Akt (PKB), mTOR, S6K, VEGF- $\alpha$ , PTEN, FoxO1, NFkB, IRS, and TSC2

RT gene expression was done following RNA extraction from the lung samples. This was followed by the synthesis of complementary DNA (cDNA) using a purified DNA-free RNA template that was extracted. Amplification of the obtained cDNA was carried out using polymerase chain reaction (PCR) technique, which involved the utilization of DNA templates as well as forward and backward primers of the gene of interest. These primers are as follow in Table 2 below.

Gel electrophoresis of the amplified genes was conducted, migrated bands were captured and the degree of intensity of each cDNA band were quantified relative to that of  $\beta$ -actin cDNA (the house-keeping gene) using Image analysis software as described by Schneider et al. [42].

## 2.10. Total protein estimation

Total protein levels in the lung samples were estimated by using the method of Gornall et al. [43], and were used for the calculation of lung antioxidant parameters.

## 2.11. Histopathology of lung sections

This was done as described by Somade et al. [44] using sections of lung fixed in phosphate-buffered formalin solution. Embedded tissues in paraffin were stained and later observed under microscope at x100 magnification.

## 2.12. Statistical analyses

One way analysis of variance (ANOVA) was used, while multiple comparisons for significance among the groups was done using Tukey's test in Graph Pad Prism Programme software version 6.0. Mean and standard error of mean (SEM) were used for the expression of results. P value greater than 0.05 (95% CI) was not taken to be significant.

**Table 2**

Primer sequences (forward (F) and reverse (R)) for the gene of interest.

Gene		Sequences
NFkB	F	5'-TCCCACAAGGGGACATTAAGC-3'
	R	5'-CAATAGGCCTCTAGTAGTAGCCC-3'
Akt	F	5'-TCATGTAGCGCACCTTCCAT-3'
	R	5'-TTCTGCAGGACAGGTTCTC-3'
PI3K	F	5'-TGGCCCGGGTAGGTTTGAAT-3'
	R	5'-ATGCCCTAGGTGACCTGACA-3'
FoxO1	F	5'-CGGCCCAATCTCGG-3'
	R	5'-CTTGCTCCCTCTGGATTGA-3'
IRS-1	F	5'-ACTGAGAGCATCACTGCCAC-3'
	R	5'-CCCCATTTCCTTTGCGGTTG-3'
S6K	F	5'-TTGGGCAATTCATCAAAGGG-3'
	R	5'-GACTCCACCAATCCACAGCA-3'
K-Ras	F	5'-GACAGGGTGTGACGATGC-3'
	R	5'-AAGTGTGCCTTAAGAAAGAGTACAA-3'
mTOR	F	5'-AGCCGGACTCCTCCACTAA-3'
	R	5'-CTGGAGGGCAAAGAGTTGCT-3'
TSC2	F	5'-GCGGGAGCAGTTCTTACCA-3'
	R	5'-ACCTAGGATTTGGCCTTGACG-3'
PDPK1	F	5'-CCACCAGCCAGCTGTATGAC-3'
	R	5'-CGGCTCTGAATGGTGAAGT-3'
PTEN	F	5'-AGACCATAACCCACCACAGC-3'
	R	5'-ACCTTTAGCTGGCAGACCAC-3'
VEGF- $\alpha$	F	5'-ACAGAAGGGGAGCAGAAAGC-3'
	R	5'-GCAACGCGAGTCTGTGTTT-3'
$\beta$ -Actin	F	5'-CCCGGAGTACAACCTTCTT-3'
	R	5'-CATCGGTAGTCCGACACAA-3'

### 3. Results

#### 3.1. Effect of SYR and ASC treatments on relative lung weight

Table 3 shows the lung weight of animals before and after administrations of NDMA, while Fig. 1 shows the results of relative lung weight of animals. In this result, there was a non-significant increase ( $p > 0.05$ ) in the relative lung weight after NDMA administration compared with control. SYR and ASC showed no significant effect on relative lung weight (Fig. 1) after treatments.

#### 3.2. Effect of SYR and ASC treatments on lung levels of MDA, NO, and GSH

Lung MDA (Fig. 2A) level was significantly ( $p < 0.05$ ) increased by NDMA administration (by 91.74%) compared with control. MDA level was brought down significantly ( $p < 0.05$ ) by the administration of SYR (by 49.65%; SYR/NDMA) and ASC (by 50.40%; ASC/NDMA) compared with NDMA only administered rats. Lung NO (Fig. 2B) was insignificantly ( $p > 0.05$ ) decreased following administration of NDMA to rats compared with control. A significant decrease ( $p < 0.05$ ) in NO level was recorded after SYR (by 30.12%; SYR/NDMA) and ASC (by 18.13%; ASC/NDMA) treatments compared with NDMA only administered rats. For lung GSH (Fig. 2C), a significant ( $p < 0.05$ ) decrease by 27.58% was recorded as a result of NDMA administration compared with control rats. SYR treatment significantly ( $p < 0.05$ ) increased GSH level by 22.97% (SYR/NDMA), but ASC (ASC/NDMA) recorded a non-significant ( $p > 0.05$ ) increase, compared with NDMA administered rats.

#### 3.3. Effect of SYR and ASC treatments on activities of lung GPx, GST, CAT, and SOD

Compared to the control, activity of lung GPx (Fig. 3A) was significantly ( $p < 0.05$ ) decreased by NDMA administration (by 32.72%). No significant effect was seen after ASC intervention (ASC/NDMA), but a significant 29.03% increase was seen following SYR (SYR/NDMA) treatment, compared with NDMA only. In Fig. 3B, there was a decrease in the activity of lung GST by 59.63% compared with the control. After treatment with SYR and ASC, there was a significant ( $p < 0.05$ ) increase in the activity of the enzyme by 212.43% (SYR/NDMA) and 431.97% (ASC/NDMA) respectively compared with NDMA only administered rats. Activity of lung CAT (Fig. 3C) was reduced (by 24.99%) significantly ( $p < 0.05$ ) by NDMA administration compared with control. Following treatments, only SYR (SYR/NDMA) was effective and increased the activity of CAT by 17.75% compared with NDMA only challenged rats. Similarly, activity of lung SOD (Fig. 3D) was reduced significantly ( $p < 0.05$ ) by NDMA administration (by 28.66%) compared with control. Following treatments, only SYR was effective and increased the activity of SOD by 23.43% (SYR/NDMA) compared with NDMA only challenged rats.

#### 3.4. Effect of SYR and ASC treatments on levels of lung TNF- $\alpha$ , IL-1 $\beta$ , caspase-3, bax, and bcl-2

TNF- $\alpha$  level in the lung of rats administered NDMA was significantly ( $p < 0.05$ ) elevated by 79.87% compared with TNF- $\alpha$  level of control animals (Fig. 4A). Following treatments, there was a significant ( $p < 0.05$ ) decrease by 38.31% for SYR (SYR/NDMA), while there was a

**Table 3**

Lung weights before and after NDMA administrations.

Lung weight (g) before NDMA administrations	Lung weight (g) after NDMA administrations
0.86 $\pm$ 0.04	1.49 $\pm$ 0.14

Values are expressed as mean  $\pm$  SEM. NDMA = N-Nitrosodimethylamine.

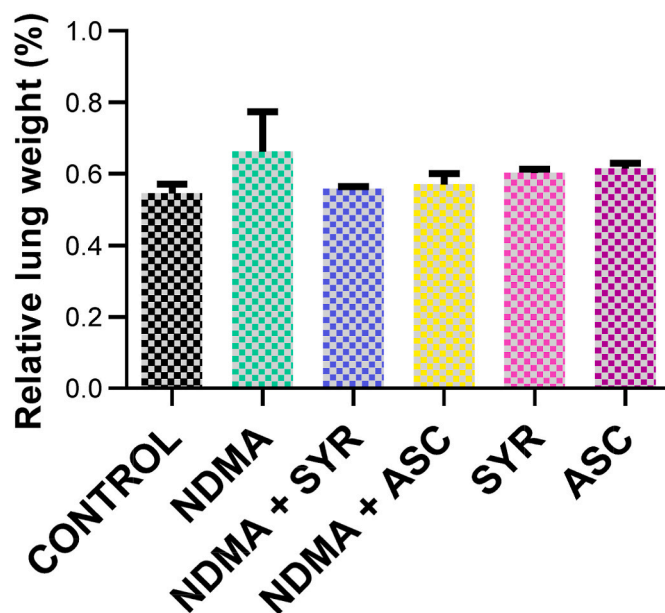


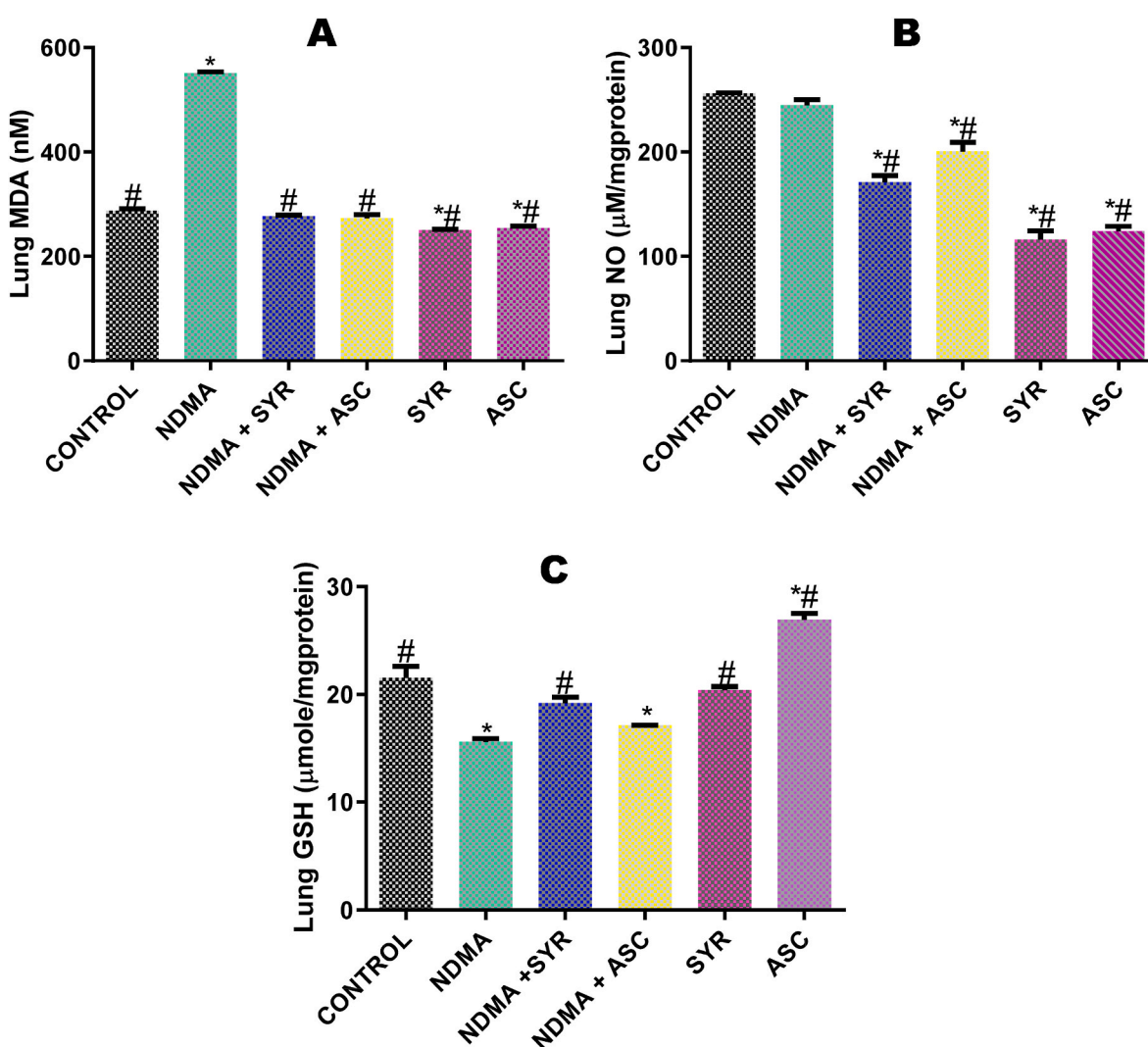
Fig. 1. Effects of SYR and ASC treatments on relative weights in NDMA-induced lung fibrosis. Bars represent the mean  $\pm$  SEM. NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.

decrease by ASC (ASC/NDMA) treatment but was not significant ( $p > 0.05$ ), when both treatments were compared with NDMA only. Similarly, lung IL-1 $\beta$  (Fig. 4B) was significantly increased ( $p < 0.05$ ) by 123.56% compared with control. Following treatment, only SYR (SYR/NDMA) was significantly effective ( $p < 0.05$ ) in lowering the level of IL-1 $\beta$  (by 18.66%) compared with NDMA only. Caspase-3 level (Fig. 4C) in the lung was also elevated ( $p < 0.05$ ) significantly by 76.60% as a result of NDMA administration compared with control. Treatments with SYR and ASC were effective, as evidenced by a significant ( $p < 0.05$ ) decrease in lung caspase-3 level by 30.18% (SYR/NDMA) and 32.67% (ASC/NDMA) respectively, compared with NDMA only. Similarly, for Bax (Fig. 4D), a significant ( $p < 0.05$ ) elevation by 55.77% in its level was recorded following NDMA administration compared with control. At the end of treatments, both treatments with SYR and ASC were potent as evidenced by the significant ( $p < 0.05$ ) decrease by 14.91% (SYR/NDMA) and 15.91% (ASC/NDMA) respectively compared with NDMA only. On the other hand, following NDMA administration, Bcl-2 level (Fig. 4E) was significantly ( $p < 0.05$ ) reduced by 57.20% compared with control. This decrease was significantly ( $p < 0.05$ ) increased after treatments with SYR by 66.22% (SYR/NDMA) and ASC by 75.57% (ASC/NDMA) compared with toxicant only group. A percentage increase of 212.55% was recorded when the Bax/Bcl-2 (Fig. 4F) ratio of NDMA group was compared with control. This significant ( $p < 0.05$ ) increase in the ratio was significantly ( $p < 0.05$ ) lowered by both SYR (by 40.91%; SYR/NDMA) and ASC (by 42.73%; ASC/NDMA) treatments compared with control.

#### 3.5. Effect of SYR and ASC treatments on immunohistochemical protein expressions of lung Nrf2

Following immunohistochemical examination, there was a significant ( $p < 0.05$ ) reduction in the protein expression of Nrf2 in the NDMA only group by 84.44% compared with control (Fig. 5A). After SYR and ASC treatments of NDMA-induced toxicity, there was a significant ( $p < 0.05$ ) elevation by 220.09% (SYR/NDMA) and 265.65% (ASC/NDMA) respectively in the Nrf2 protein expressions compared with NDMA only (Fig. 5A).





**Fig. 2.** Effects of SYR and ASC treatments on levels of MDA (A), NO (B), and GSH (C) in NDMA-induced lung fibrosis. Bars represent the mean  $\pm$  SEM. \*significantly different compared with control ( $p > 0.05$ ); #significantly different compared with NDMA only. NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.

### 3.6. Effect of SYR and ASC treatments on immunohistochemical protein expressions of lung collagen-1 and $\alpha$ -SMA

Collagen-1 level in the lung was significantly ( $p < 0.05$ ) increased by 734.92% after NDMA administration compared with control (Fig. 5B). Treatments of this lung toxicity by SYR and ASC were significant ( $p < 0.05$ ) judging by the reduction in collagen-1 level by 72.82% (SYR/NDMA) and 49.35% (ASC/NDMA) respectively compared with the toxicant only (Fig. 5B). Similarly, lung  $\alpha$ -SMA (Fig. 5C) expression was significantly ( $p < 0.05$ ) increased by 113.51% in the NDMA only group compared with control. After treatments with SYR and ASC (Fig. 5C),  $\alpha$ -SMA protein expression was significantly ( $p < 0.05$ ) brought down by 30.71% (SYR/NDMA) and 48.98% (ASC/NDMA) respectively compared with NDMA only administered rats.

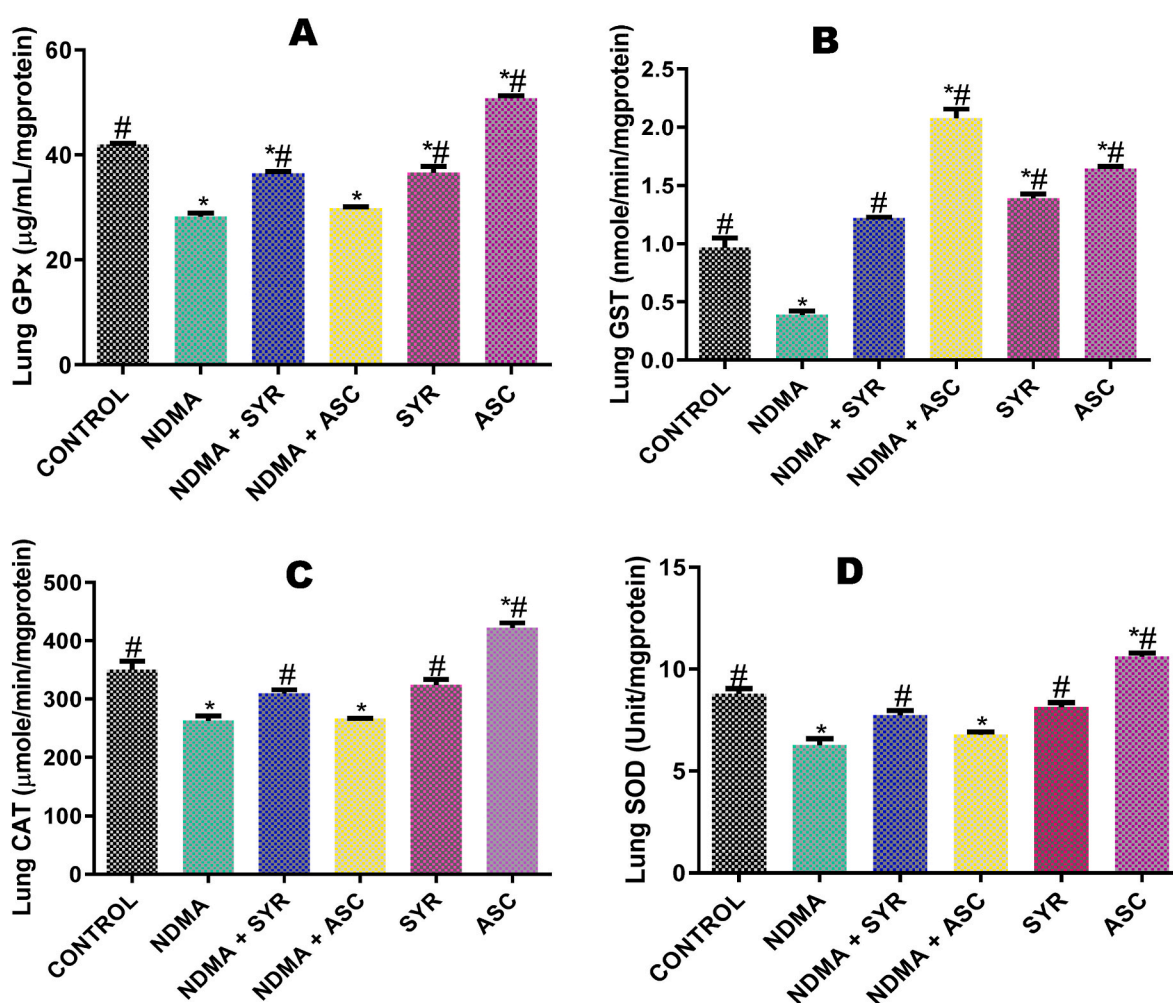
### 3.7. Effect of SYR and ASC treatments on immunohistochemical protein expressions of lung p53, mdm2 and cyclin D1

NDMA administration significantly ( $p < 0.05$ ) increased the protein expression of lung tumor suppressor p53 (Fig. 5D) by 375.46% compared with control. This elevation in the p53 level was brought down by SYR (63.88%; SYR/NDMA) and ASC (43.16%; ASC/NDMA) treatments compared with NDMA only (Fig. 5D). On the other hand, the

protein expressions of both mdm2 (Fig. 5E) and cyclin D1 (Fig. 5F) in the NDMA only group were significantly ( $p < 0.05$ ) decreased by 84.83% and 91.04% respectively compared with control. Both treatments with SYR and ASC were highly effective in increasing the expressions of the two proteins. SYR significantly ( $p < 0.05$ ) increased the expressions of mdm2 (Fig. 5E) and cyclin D1 (Fig. 5F) by 1656.12% (SYR/NDMA) and 852.94% (SYR/NDMA) respectively, while ASC significantly ( $p < 0.05$ ) increased the expressions of mdm2 (Fig. 5E) and cyclin D1 (Fig. 5F) by 1046.94% (ASC/NDMA) and 280.54% (ASC/NDMA) respectively compared with NDMA only.

### 3.8. Effect of SYR and ASC treatments on mRNA expressions of lung K-ras, PI3K, PDPK1, Akt and NFkB

Compared with control, administration of NDMA to rats significantly ( $p < 0.05$ ) decreased the mRNA expression level of Kras (Fig. 6A) by 20.16%, and significantly ( $p < 0.05$ ) increased the mRNA expression of PI3K (Fig. 6B) by 12.82%. mRNA expression level of PDPK1 (Fig. 6C) was also significantly ( $p < 0.05$ ) decreased by 12.90%, while expression levels of Akt (Fig. 6D) and NFkB (Fig. 6E) were significantly ( $p < 0.05$ ) elevated by 7.96% and 50.33% respectively. Following treatment with SYR, mRNA expression level of Kras was significantly ( $p < 0.05$ ) increased by 8.16% (SYR/NDMA), while it was significantly ( $p < 0.05$ )



**Fig. 3.** Effects of SYR and ASC treatments on activities of GPx (A), GST (B), CAT (C), and SOD (D) in NDMA-induced lung fibrosis. Bars represent the mean  $\pm$  SEM. \*significantly different compared with control ( $p > 0.05$ ); #significantly different compared with NDMA only ( $p > 0.05$ ). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.

decreased by ASC (35.24%; ASC/NDMA) compared with NDMA only (Fig. 6A). PI3K mRNA expression was significantly ( $p < 0.05$ ) reduced by SYR (42.29%; SYR/NDMA) and ASC (32.88%; ASC/NDMA) treatments compared with NDMA only (Fig. 6B). PDPK1 expression on the other hand, was significantly ( $p < 0.05$ ) decreased by SYR (11.71%; SYR/NDMA), and increased ( $p < 0.05$ ) by ASC (16.77%; ASC/NDMA) compared with toxicant only (Fig. 6C). For both SYR and ASC treatments, there was a significant ( $p < 0.05$ ) decrease in the mRNA levels of Akt by 48.20% (SYR/NDMA) and 33.61% (ASC/NDMA) respectively (Fig. 6D), while for NFkB, the significant ( $p < 0.05$ ) decrease was by 6.50% (SYR/NDMA) and 10.95% (ASC/NDMA) respectively compared with the toxicant only (Fig. 6E).

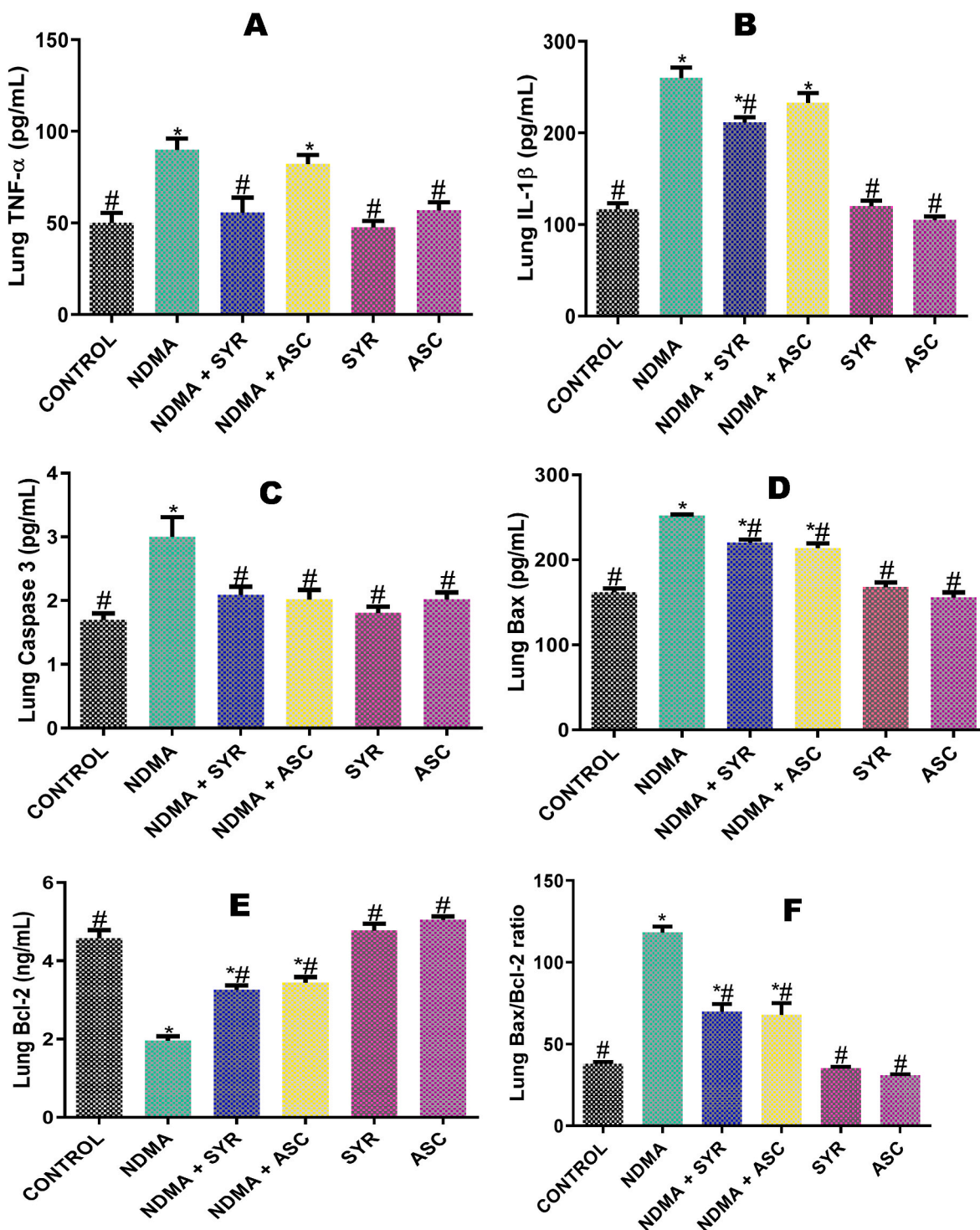
### 3.9. Effect of SYR and ASC treatments on mRNA expressions of lung mTOR, S6K, IRS-1 and VEGF- $\alpha$

mRNA expression level of mTOR was significantly ( $p < 0.05$ ) increased by 7.45% following the administration of NDMA in rats compared with control (Fig. 7A). This expression was significantly ( $p < 0.05$ ) reduced by 27.21% and 13.66% following treatments with SYR (SYR/NDMA) and ASC (ASC/NDMA) respectively compared with NDMA only (Fig. 7A). Administration of NDMA did not have a significant ( $p > 0.05$ ) effect on mRNA expression of S6K compared with control, but a significant ( $p < 0.05$ ) increase was seen only after SYR treatment (by 8.18%; SYR/NDMA) compared with NDMA alone

(Fig. 7B). Elsewhere, the mRNA levels of both IRS-1 (Fig. 7C) and VEGF- $\alpha$  (Fig. 7D) were significantly ( $p < 0.05$ ) decreased by the administration of NDMA to rats by 28.62% and 25.00% respectively compared with control. For IRS-1, it was only SYR that further reduced the expression of IRS-1 significantly ( $p < 0.05$ ) by 16.33% (SYR/NDMA) compared with NDMA alone (Fig. 7C), while both SYR and ASC were able to further decrease the mRNA level of VEGF- $\alpha$  significantly by 6.48% (SYR/NDMA) and 11.03% (ASC/NDMA) respectively compared with NDMA only (Fig. 7D).

### 3.10. Effect of SYR and ASC treatments on mRNA expressions of lung PTEN, FoxO1 and TSC2

Administration of NDMA to rats significantly ( $p < 0.05$ ) decreased the mRNA expression levels of PTEN, FoxO1, and TSC2 by 37.38% (Fig. 8A), 28.41% (Figs. 8B), and 16.14% (Fig. 8C) respectively, compared with control. After treatments, both SYR and ASC significantly ( $p < 0.05$ ) increased PTEN mRNA expression by 82.49% (SYR/NDMA) and 84.60% (ASC/NDMA) respectively compared with the toxicant only (Fig. 8A). For FoxO1 level, only treatment with ASC was effective in lowering it by 22.22% (ASC/NDMA) compared NDMA alone (Fig. 8B), while for TSC2, it was only SYR that significantly ( $p < 0.05$ ) increased the expression by 10.16% (SYR/NDMA) also when compared with NDMA only (Fig. 8C).



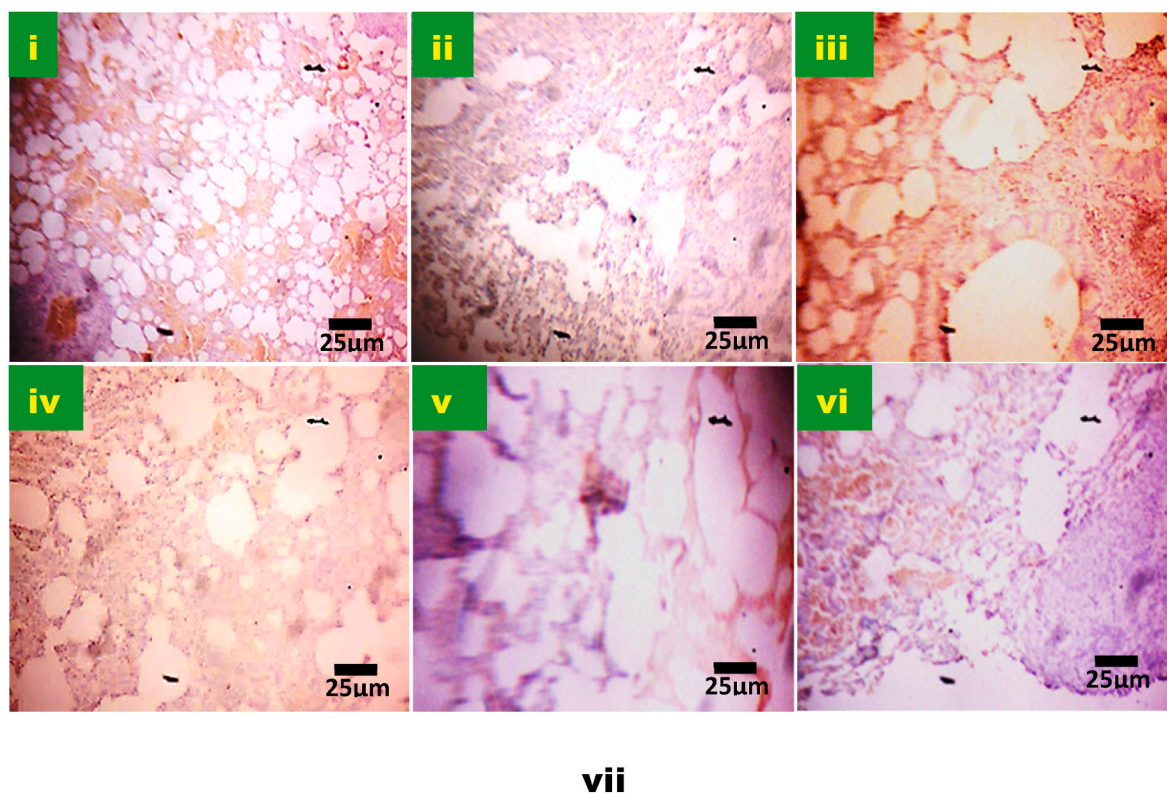
**Fig. 4.** Effects of SYR and ASC treatments on levels of pro-inflammatory (TNF- $\alpha$  and IL-1 $\beta$ ) and apoptotic (caspase-3, bax, and bcl-2) markers in NDMA-induced lung fibrosis. Bars represent the mean  $\pm$  SEM. \*significantly different compared with control ( $p > 0.05$ ); #significantly different compared with NDMA only ( $p > 0.05$ ). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.

### 3.11. Effects of SYR and ASC treatments on lung alveolar and bronchi architecture

Bronchi and alveolar of control rats appeared normal with no visible lesions. NDMA administration led to disruption of architecture, alveolar thickening, hemorrhage, infiltration of inflammatory cells, and hyperplasia of the alveolar (Fig. 9A). In the bronchi, severe infiltration by inflammatory cells were also seen, and presence of very severe

hemorrhage and disrupted epithelial cells (Fig. 9B). After SYR and ASC (ASC/NDMA) intervention, there was an intact alveolar architecture with mild infiltration of inflammatory cells (SYR/NDMA) and mild alveolar hyperplasia, hemorrhage with few inflammatory cells (Fig. 9A). In the bronchi (Fig. 9B), a mild to moderate infiltration of inflammatory cells and intact bronchi epithelium were seen following SYR (SYR/NDMA) treatment, while ASC treatment (ASC/NDMA) showed moderate hemorrhage with few infiltrations of inflammatory cell and slight





**Fig. 5A.** Effects of SYR and ASC treatments on immunohistochemical expressions of p53 in NDMA-induced pulmonary fibrosis at 100× magnification. i = control; ii = NDMA only; iii = NDMA + SYR; iv = NDMA + ASC; v = SYR; vi = ASC; vii = graph showing percentage p53 positivity; each bar represents mean  $\pm$  SEM. \*significantly different compared with control ( $p > 0.05$ ); #significantly different compared with NDMA only ( $p > 0.05$ ). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.

disruption of the bronchial epithelium.

#### 4. Discussion

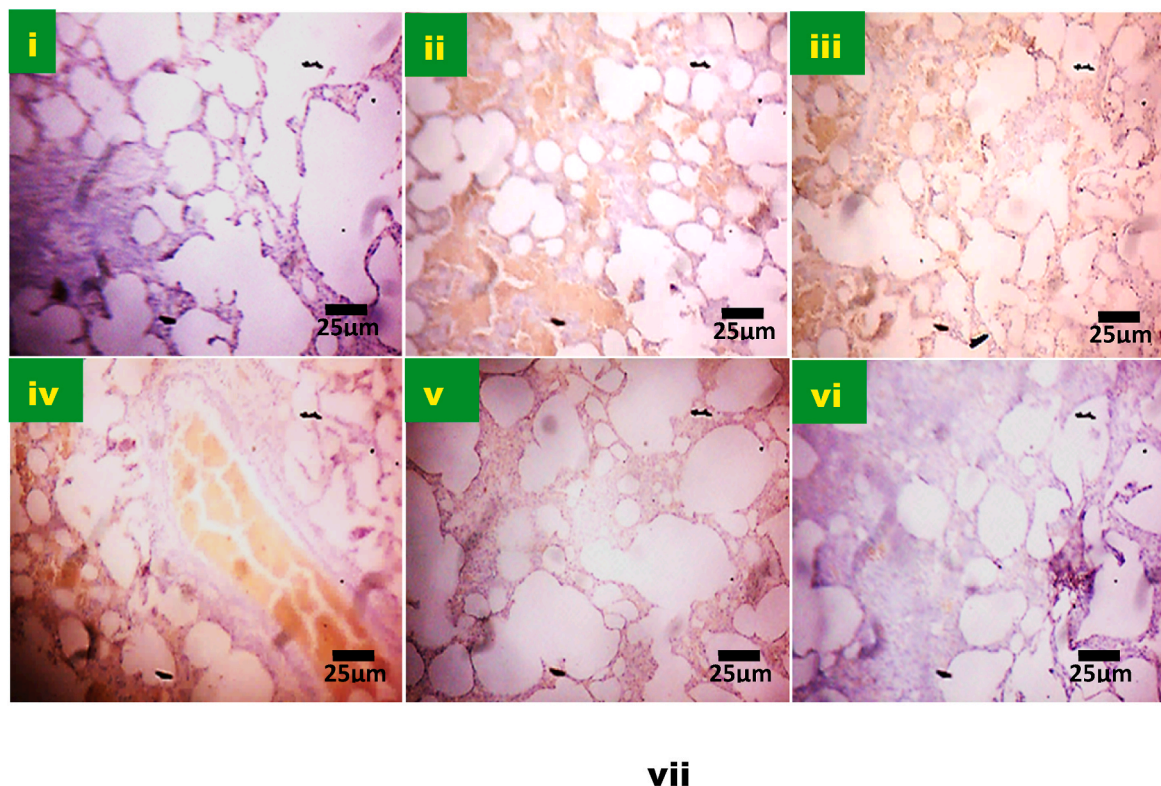
Idiopathic lung fibrosis (ILF) is a severe and life threatening lung disorder that is still characterized by poor pathophysiology and prognosis [45]. The common outcomes of ILF in patients are development of honeycomb-like lung, which can lead to uncontrolled loss of lung function [44]. In ILF, there is a formation of both fibroblast and myofibroblast foci, leading to the production of fibrillary collagens, which are examples of extracellular matrix (ECM), causing their accumulation, scarring and damage to lung architecture [46]. This study therefore investigated the effects of both SYR and ASC treatments on NDMA-induced pulmonary fibrosis, and the roles played by PI3K-Akt-mTOR signaling pathway in rats.

Features marking the pathophysiology of lung fibrosis are damage to pulmonary tissues, their apoptosis, increased proliferation of lung fibroblasts, as well as over secretion and deposition of collagen matrix [47] and  $\alpha$ -SMA [48]. Our findings in this study are not different from these, which were marked by the mass deposition of collagen type 1 and  $\alpha$ -SMA in the lung tissues of rats. Administration of NDMA to the rats may have led to the activation of the lung fibroblast causing it to metamorphose to myofibroblasts that resulted into the accumulation of these extracellular matrix proteins, and the replacement of normal lung tissues with scarred tissues [49]. Administrations of SYR and ASC however protected against these deposition of collagen-1 and  $\alpha$ -SMA. The antioxidant and cytoprotective properties of these two compounds may have conferred this protection on the lung tissues. High levels of collagen type 1 and  $\alpha$ -SMA have also been reported to accumulate in lung tissues in bleomycin-induced pulmonary fibrosis [48], and SYR has been reported to lower the gene expressions of collagen type 1 and  $\alpha$ -SMA in carbon tetrachloride-induced liver injury [50].

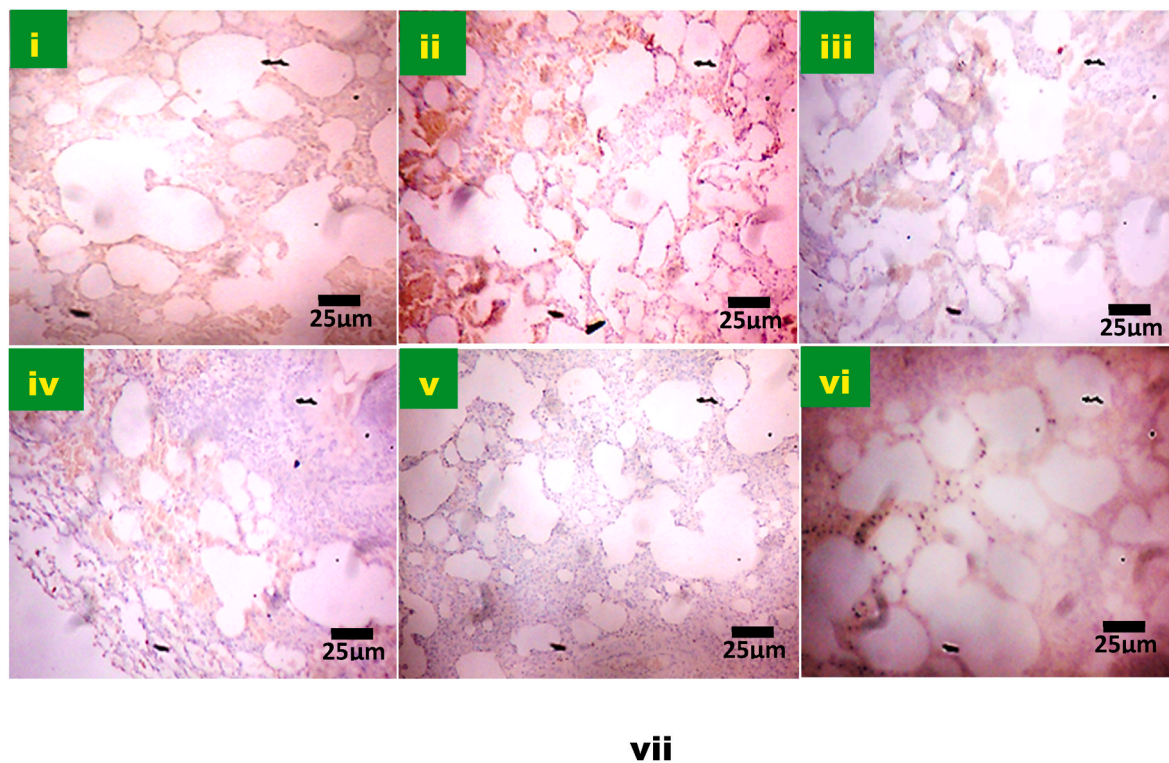
PI3K-Akt/PKB signaling pathway is among the major and most important cellular pathways that manage cell metabolism, proliferation, growth, and survival [51]. Studies have reported and linked the over-expression of  $\alpha$ -SMA in pulmonary fibrosis to activation of PI3K-Akt pathway [52]. A past study reported that Akt-null mice were protected against lung fibrosis and inflammation induced by bleomycin [53], pointing to the fact that PI3K-Akt signaling is crucial to ILF development. Our findings in this study are not different, judging by the increased levels of lung relative mRNA expressions of PI3K and Akt, implicating the activation of the PI3K-Akt signaling pathway in NDMA-induced pulmonary fibrosis. Both treatments (SYR and ASC) were observed to decrease the expressions of lung PI3K and Akt, which may suggest their ability to prevent the activation of the signaling pathway. This ability may be due to their rich antioxidant and anti-inflammatory prowess as previously documented [54–57]. PDPK1 has been established to phosphorylate Akt at T308, a site which is necessary for the activity of Akt [58]. Though, lung mRNA expression levels of PI3K and Akt were significantly elevated in this study, otherwise is the case of PDPK1 level, suggesting that the phosphorylation and activation of Akt may not be PDPK1-dependent.

mTOR function is executed by two various complexes namely mTOR complexes 1 and 2 (mTORC1 and mTORC2) [59]. It has been established that Akt is phosphorylated by mTORC2, thereby enhancing the process of cell survival and proliferation. In this study, we recorded a significant increase in lung mRNA expression of mTOR following NDMA administration, a signal that NDMA administrations have tendencies to stimulate lung cell proliferation and their survival. Also from the information provided above, the significant high level of mTOR expression in this study also suggests that mTORC2 and not PDPK1 may be responsible for the phosphorylation and activation of Akt. Also, from the report of Lawrence and Nho [60], mTOR is said to play a pivotal role in regulating metabolic pathways that positively influence pulmonary fibrogenesis.



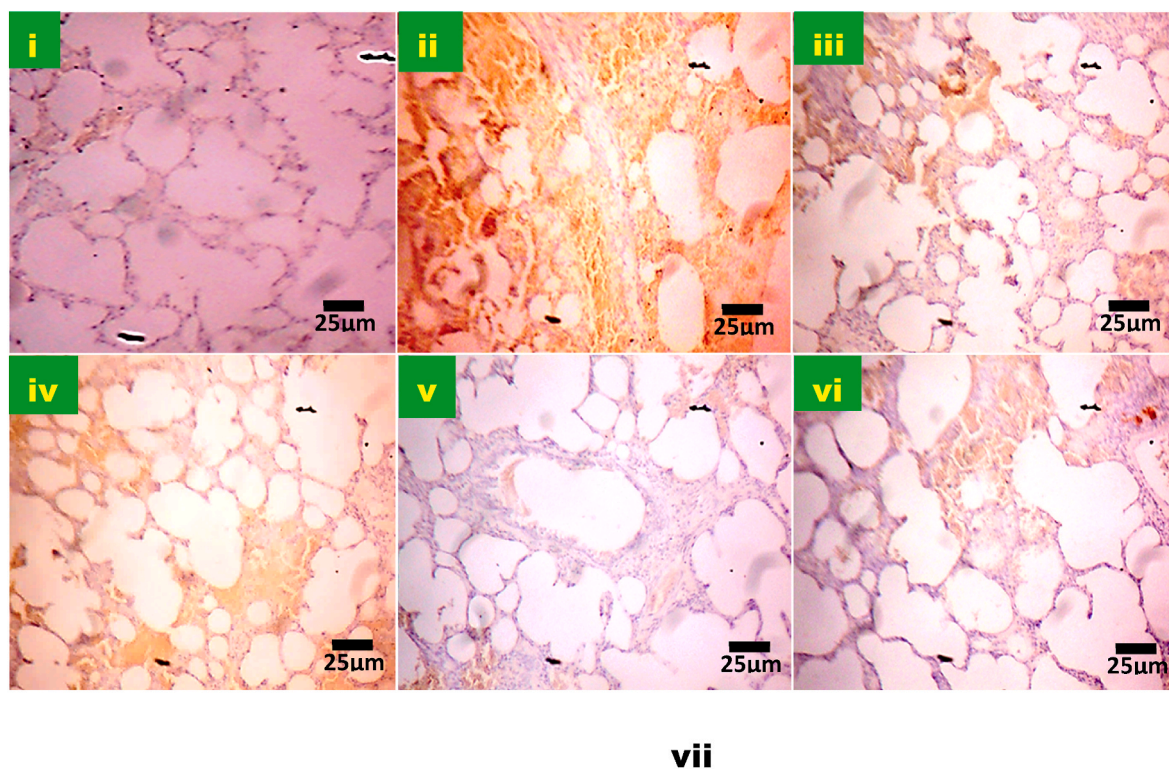


**Fig. 5B.** Effects of SYR and ASC treatments on immunohistochemical expressions of mdm2 in NDMA-induced pulmonary fibrosis at 100× magnification. i = control; ii = NDMA only; iii = NDMA + SYR; iv = NDMA + ASC; v = SYR; vi = ASC; vii = graph showing percentage mdm2 positivity; each bar represents mean ± SEM. \*significantly different compared with control ( $p > 0.05$ ); #significantly different compared with NDMA only ( $p > 0.05$ ). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.

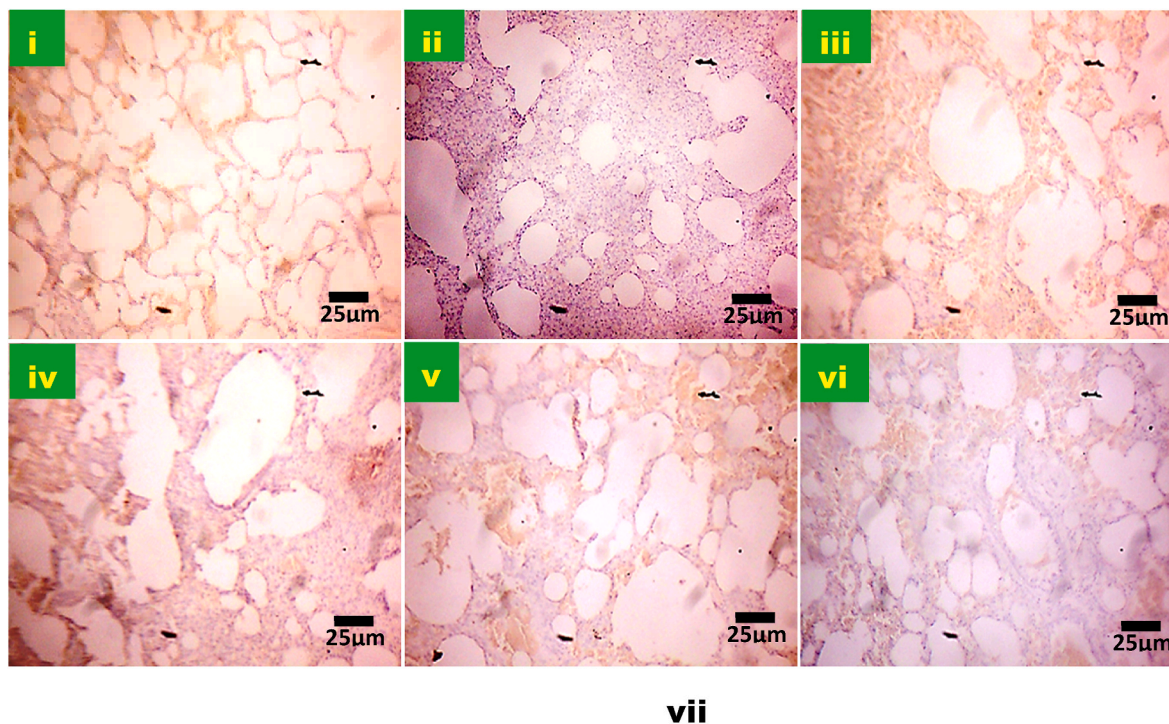


**Fig. 5C.** Effects of SYR and ASC treatments on immunohistochemical expressions of collagen-1 in NDMA-induced pulmonary fibrosis at 100× magnification. i = control; ii = NDMA only; iii = NDMA + SYR; iv = NDMA + ASC; v = SYR; vi = ASC; vii = graph showing percentage collagen-1 positivity; each bar represents mean ± SEM. \*significantly different compared with control ( $p > 0.05$ ); #significantly different compared with NDMA only ( $p > 0.05$ ). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.



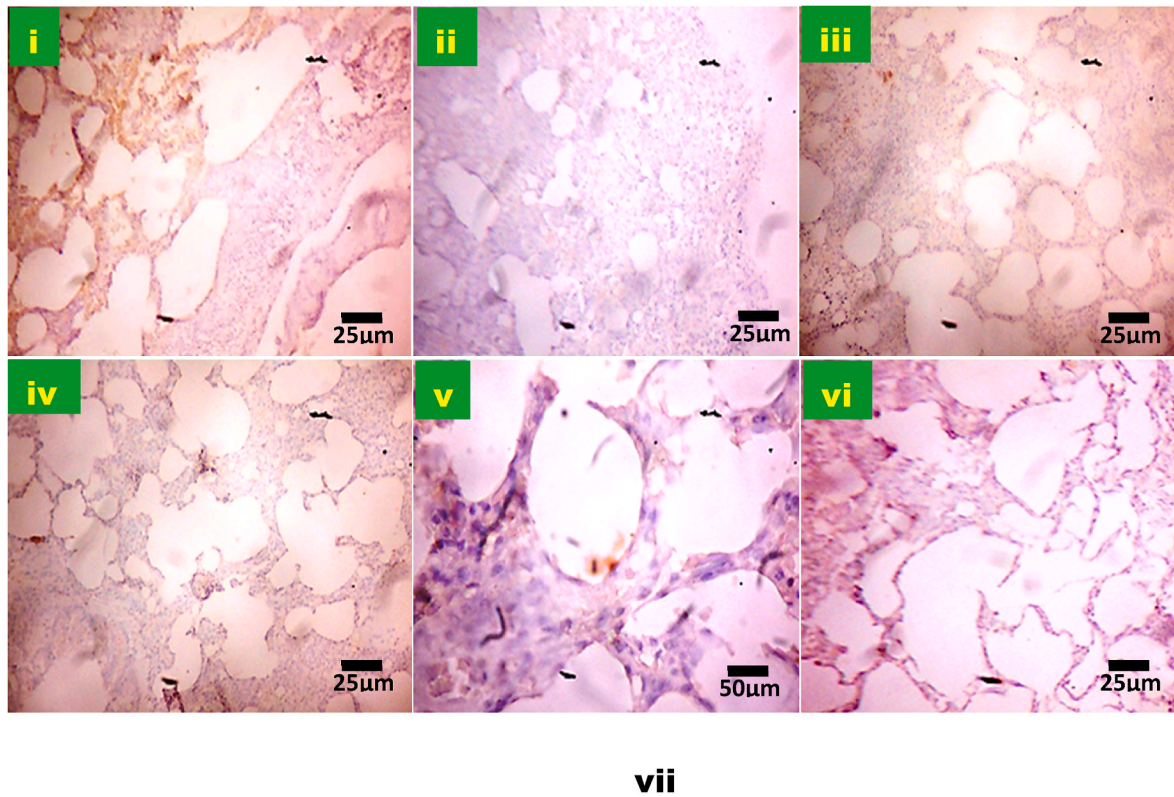


**Fig. 5D.** Effects of SYR and ASC treatments on immunohistochemical expressions of  $\alpha$ -SMA in NDMA-induced pulmonary fibrosis at 100 $\times$  magnification. i = control; ii = NDMA only; iii = NDMA + SYR; iv = NDMA + ASC; v = SYR; vi = ASC; vii = graph showing percentage  $\alpha$ -SMA positivity; each bar represents mean  $\pm$  SEM. \*significantly different compared with control ( $p > 0.05$ ); #significantly different compared with NDMA only ( $p > 0.05$ ). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.

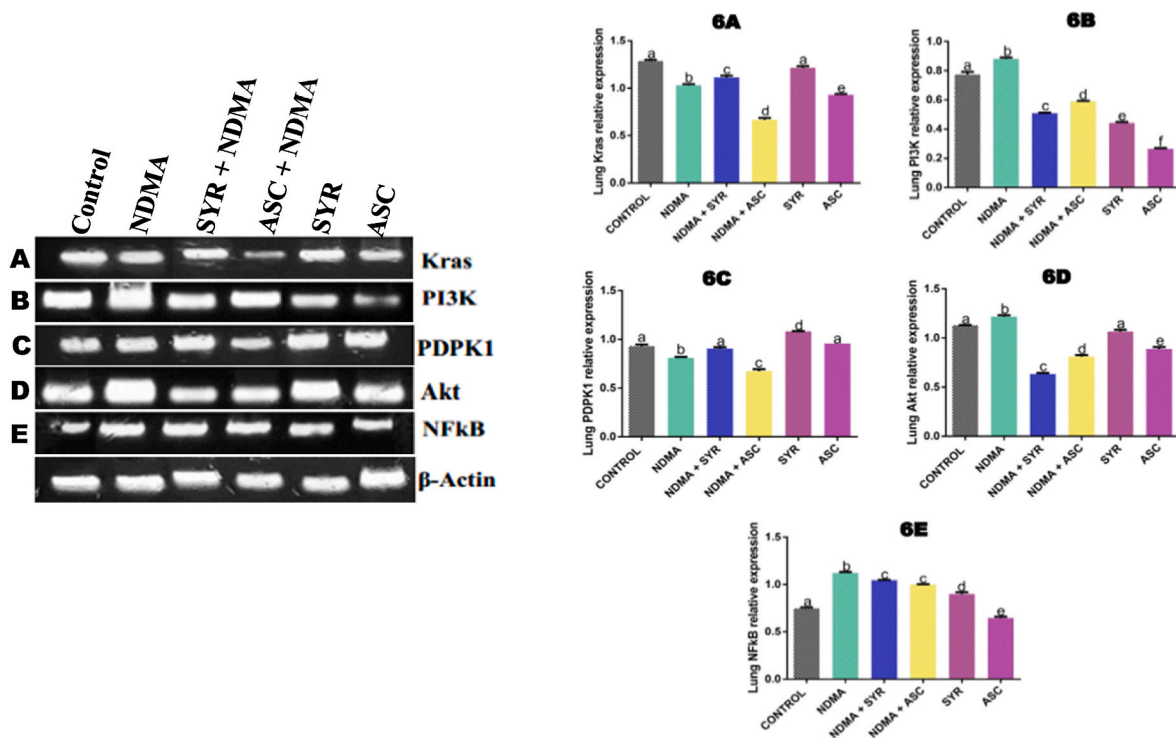


**Fig. 5E.** Effects of SYR and ASC treatments on immunohistochemical expressions of Nrf-2 in NDMA-induced pulmonary fibrosis at 100 $\times$  magnification. i = control; ii = NDMA only; iii = NDMA + SYR; iv = NDMA + ASC; v = SYR; vi = ASC; vii = graph showing percentage Nrf-2 positivity; each bar represents mean  $\pm$  SEM. \*significantly different compared with control ( $p > 0.05$ ); #significantly different compared with NDMA only ( $p > 0.05$ ). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.

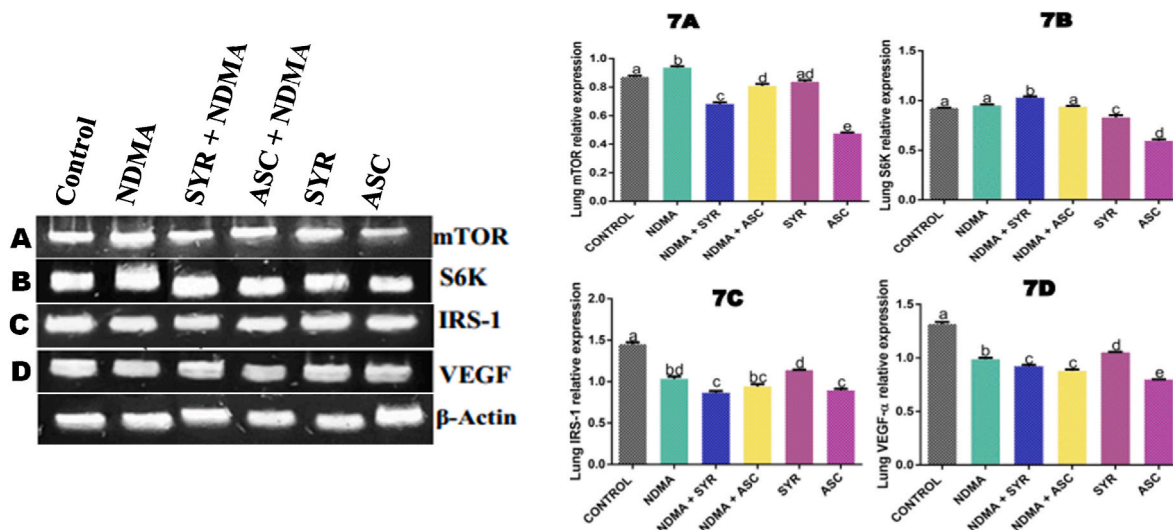




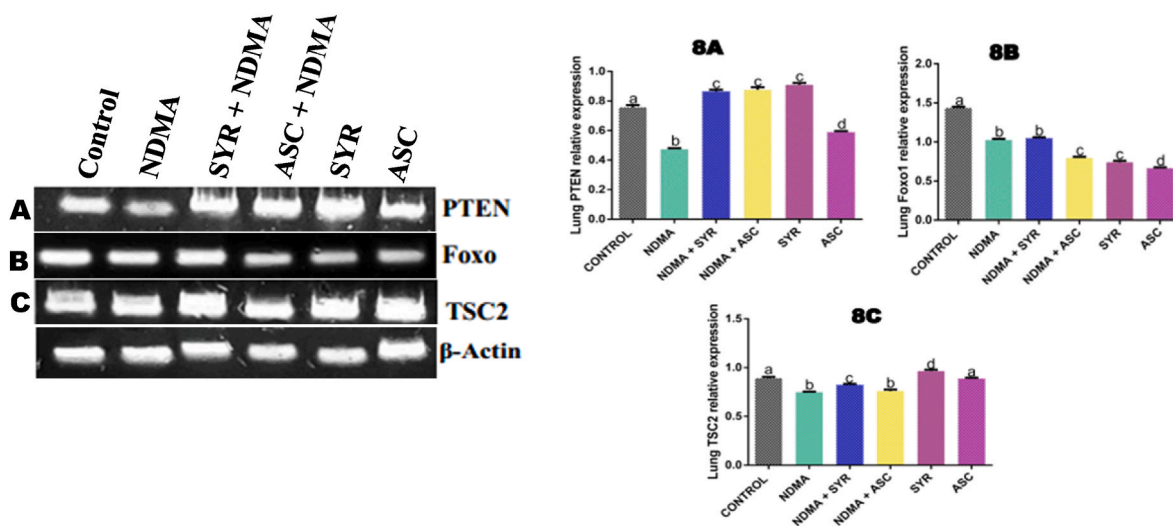
**Fig. 5F.** Effects of SYR and ASC treatments on immunohistochemical expressions of cyclin D1 in NDMA-induced pulmonary fibrosis at 100× magnification. i = control; ii = NDMA only; iii = NDMA + SYR; iv = NDMA + ASC; v = SYR; vi = ASC; vii = graph showing percentage cyclin D1 positivity; each bar represents mean ± SEM. \*significantly different compared with control (p > 0.05); #significantly different compared with NDMA only (p > 0.05). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.



**Fig. 6.** Effects of SYR and ASC treatments on relative mRNA expressions of Kras (A), PI3K (B), PDPK1 (C), Akt (D), and NFkB (E) in NDMA-induced pulmonary fibrosis. NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid. Bars represent the mean ± SEM. \*significantly different compared with control (p > 0.05); #significantly different compared with NDMA only (p > 0.05). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.



**Fig. 7.** Effects of SYR and ASC treatments on relative mRNA expressions of mTOR (A), S6K (B), IRS-1 (C) and VEGF (D) in NDMA-induced pulmonary fibrosis. NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid. Bars represent the mean ± SEM. \*significantly different compared with control ( $p > 0.05$ ); #significantly different compared with NDMA only ( $p > 0.05$ ). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.



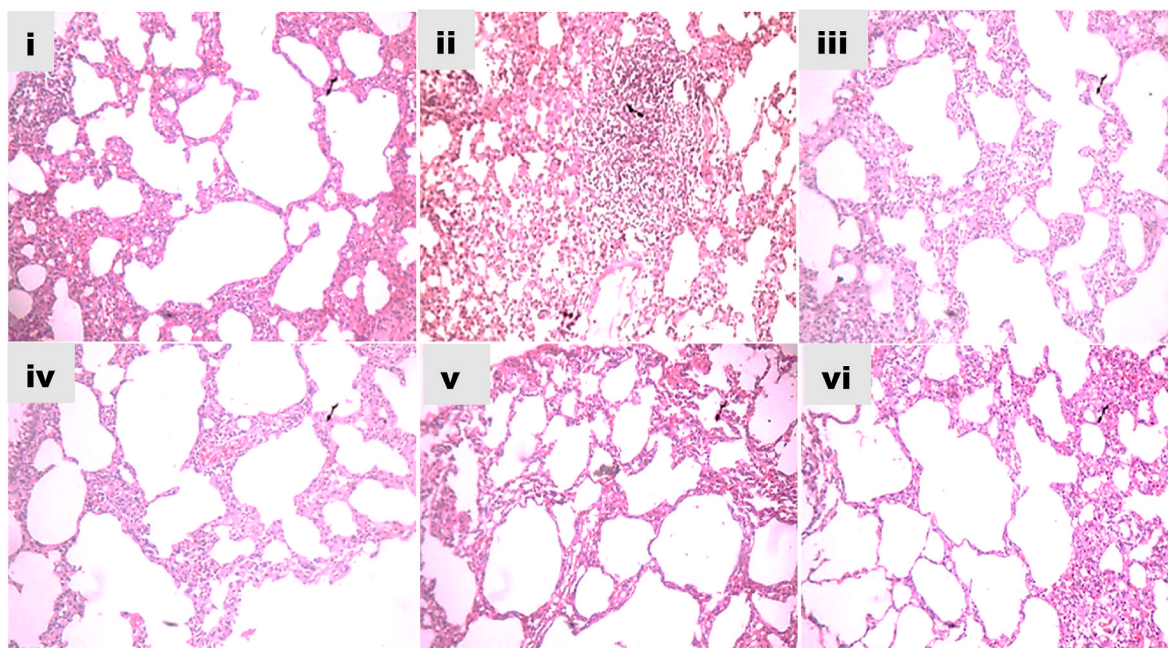
**Fig. 8.** Effects of SYR and ASC treatments on relative mRNA expressions of PTEN (A), Foxo1 (B) and TSC2 (C) in NDMA-induced pulmonary fibrosis. NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid. Bars represent the mean ± SEM. \*significantly different compared with control ( $p > 0.05$ ); #significantly different compared with NDMA only ( $p > 0.05$ ). NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.

The significant reduction in mTOR level following treatments with SYR and ASC can also be attributed to their antioxidant, anti-inflammatory, free radical scavenging and cytoprotective abilities [57,61] that may have blocked the activated mTOR to initiate the lung cell proliferation and survival. mTORC1, S6K and other protein kinases that are downstream players, promote IRS1/2 phosphorylation and activation, targeting IRS1 for degradation and thereby lessening the activation of PI3K [62]. Therefore, in this study, the increased level of mTOR mRNA after NDMA administration may be responsible for the significant decrease in lung IRS1 expression. S6K is a downstream effector of mTOR that is phosphorylated by the latter to stimulate protein translation responsible for cell growth and proliferation [63]. From the results gathered in this study, low level of lung S6K was recorded and this may also be the reason for the significantly low mRNA expression levels of IRS1. VEGF is also another downstream player of the mTOR signaling pathway, an angiogenic factor that participates in angiogenesis. From this study, administration of NDMA did not have effect on the expression of VEGF, and this may be due to the short period of exposure in this study (28

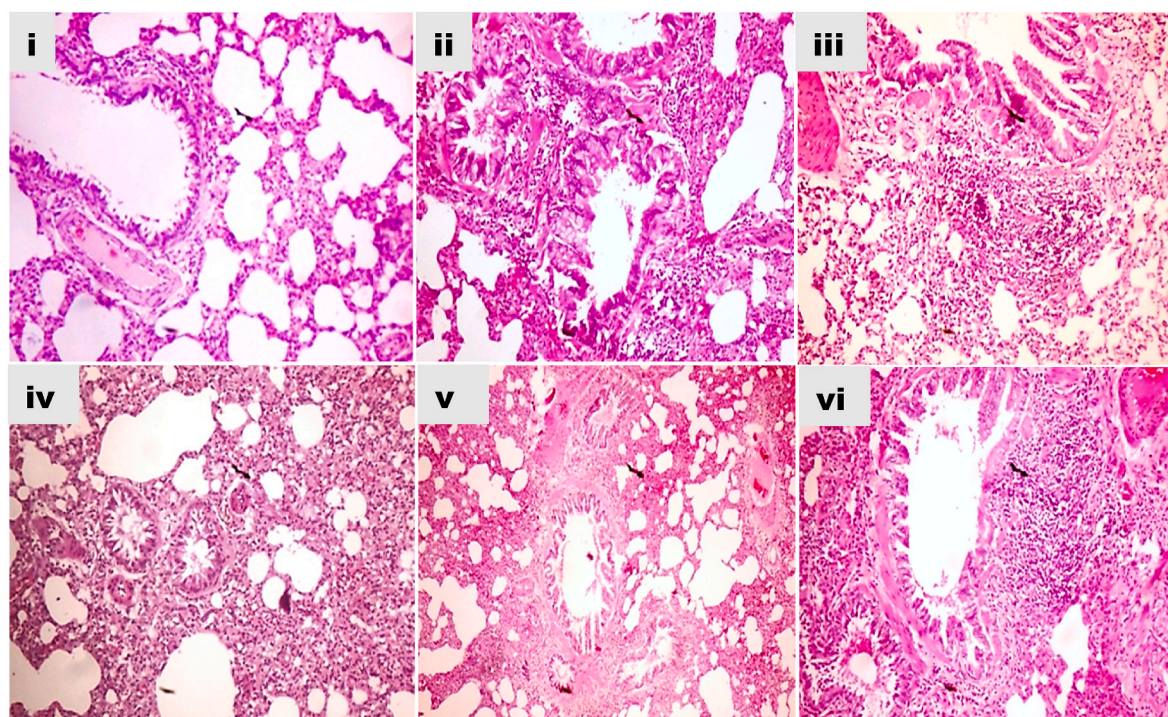
days) that has not warranted angiogenesis, a characteristic of transformed or cancer cells [64].

PTEN is a tumor suppressor gene capable of stopping fibroblast proliferation and promoting cellular apoptosis [65]. It is an important negative effector of the PI3K signaling pathway that can stop or block the phosphorylation of PIP2 to PIP3 and then inhibit the activation Akt and other downstream kinases [66]. Low mRNA expression level of PTEN was recorded in this study. This is as a result of NDMA administrations which favor the activation of both PI3K and Akt, thereby overwhelming PTEN expression. This further proved that exposure to NDMA is capable of inducing lung cancer via the suppression of PTEN as well as activation of PI3K-Akt-mTOR signaling pathway. It has been reported that in ILF patients, there was a resistance to stress-induced apoptosis resulting from an abnormally high activation of the PI3K-Akt-mTOR signaling, due to PTEN downregulation [67]. Decline in PTEN expression and high levels of Akt have also been seen in biopsy specimens of ILF patients [68]. Again, high level of lung PTEN mRNA expression that was recorded after treatments with SYR and ASC is a





**Fig. 9A.** Effects of SYR and ASC treatments on lung alveolar architecture (magnification x100). Control (i) showing no visible lesion; NDMA (ii) showing disrupted architecture, alveolar thickening, hemorrhage, infiltration of inflammatory cells, and alveolar hyperplasia; NDMA + SYR (iii) showing intact alveolar architecture with mild infiltration of inflammatory cells; NDMA + ASC (iv) showing mild alveolar hyperplasia, mild hemorrhage and with few inflammatory cells; SYR (v) showing no visible lesion; ASC (vi) showing no visible lesion. NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.



**Fig. 9B.** Effects of SYR and ASC treatments on lung bronchi architecture (magnification x100). Control (i) showing no visible lesion; NDMA (ii) showing severe infiltration by inflammatory cells of the bronchi, small vascular submucosa, and surrounding tissues. There is presence of very severe hemorrhage and disrupted epithelial cells; NDMA + SYR (iii) showing mild to moderate infiltration of inflammatory cells. The bronchi epithelium is intact; NDMA + ASC (iv) showing moderate hemorrhage with few inflammatory cell infiltration and slight disruption of the bronchial epithelium; SYR (v) showing no visible lesion; ASC (vi) showing no visible lesion. NDMA = N-Nitrosodimethylamine; SYR = syringic acid; ASC = ascorbic acid.

proof that both compounds have antioxidant, anti-apoptotic and cell-protecting abilities, capable of blocking the activities of activated PI3K and Akt, and surmounting tumor development via the induction of apoptosis. In this study, the proposed mechanism of action of SYR and

ASC is via PTEN-induced inhibition of PI3K and Akt. Some of other compounds that have been previously documented to have PI3K-Akt-mTOR inhibition in ILF are quercetin [69], LY294002 [70], and wortmannin [70].

Another inhibitor in the PI3K-Akt-mTOR signaling pathway is TSC2. PI3K-Akt signaling is associated with cellular growth and its survival by interacting with TSC1/2 along mTOR pathway and also, via the inhibition of pro-apoptotic players or signals [71]. Activation of Akt blocks the TSC1/2 complex formation, allowing Rheb to activate mTORC1. Following Akt phosphorylation of TSC2, there is dissociation of the TSC1-TSC2 complex, causing mTORC1 activation. mTORC1 activation therefore inhibits autophagy [72]. Similarly, NDMA-induced significant reduction in TSC2 mRNA expression level was observed. This observation may be due to NDMA-induced Akt activation, which can phosphorylate and inactivate TSC2 that is supposed to check mTORC1 activity through the inhibition of Rheb, a direct activator of mTORC1. The inhibition of TSC2 as a result of Akt phosphorylation may therefore be responsible for the significant high level of mTOR mRNA level recorded in this study. SYR only was able to increase the TSC2 expression significantly and therefore, may serve as a good candidate for blocking mTOR activity. Mechanism of action of SYR may be by its ability to stimulate TSC2 to inhibit Rheb, after PTEN inhibition of PI3K-Akt pathway.

Nrf2 is a transcription factor that regulates the nuclear transcription of many genes coding for antioxidant proteins that are needed to abrogate oxidative stress [73,74]. Nrf2 is among the downstream target of Akt, and the interplay of PI3K-Akt and Nrf2 signaling pathways superintends over the defense system of cells, guarding against tissue oxidative and inflammatory damage [75]. Depletion of Nrf2 protein expression level was observed in this study. This may be attributed to NDMA-induced over generation of reactive species that were capable of overwhelming Nrf2 expression. The over generated reactive species were surmounted by both SYR and ASC treatments, thereby sparing and restoring the lung Nrf2 level. These effects of SYR and ASC are due to their antioxidant and free radical scavenging properties. Oxidative stress has also been linked to the development of lung fibrosis. The imbalance between pro-oxidative and anti-oxidative state may initiate epithelial cells apoptosis and the activation of fibrogenesis [76]. In this study, NDMA-induced lung lipid peroxidation and oxidative stress marked by elevated level of MDA, and decreased level of GSH, as well as activities of CAT, SOD, GST and GPx were seen, which is attributed to free radical formation as previous reported [44,57,77]. Lung lipid peroxidation and oxidative stress were suppressed by SYR and ASC treatments. This anti-oxidative property of both compounds has been linked to their free radical scavenging (antioxidant) abilities as reported by Rashedinia et al. [78], Cikman et al. [79], Somade et al. [56] and Somade et al. [57]. High level of Nrf2 recorded following treatments with SYR and ASC may be responsible for the restoration of the endogenous antioxidant systems in the animals.

NFκB is a transcription factor that is known to play a significant role in the regulation of inflammation and immune responses [80]. One of the signaling pathways linked to NFκB signaling is the PI3K-Akt [81]. Akt has numerous downstream targets, one of which is IκB kinase (IKK) [82]. NFκB is usually sequestered with IκB in the cytoplasm in an unstressed state. Following stress, IKK is activated and phosphorylate IκB, thereby stopping their sequestration. Upon separation, NFκB becomes activated and translocate to the nucleus where it exerts its nuclear transcriptional role [40,41]. In this study, NDMA-induced NFκB mRNA expression was observed. This is unsurprising as NFκB is one of the downstream targets of the PI3K-Akt pathway, and the proposed mode of NFκB activation by Akt may be by the mechanism described above, i.e via Akt phosphorylation of IKK. The significant decrease in the mRNA expression of NFκB after treatments with SYR and ASC is an indication and proof of their cyto-protective and anti-inflammatory properties that make them capable of checking and maintaining NFκB in an inactive state, which may be through inhibition of IKK phosphorylation by Akt [83]. Studies have shown that tissue injuries are marked by inflammation characterized by production of pro-inflammatory cytokines like IL-1β and TNF-α [40]. Production of these cytokines poses harmful effects at the site of formation, and that is why studies have focused on

these cytokines in understanding and treating tissue inflammation [84]. Elevated levels of TNF-α and IL-1β are indications of NDMA-induced lung inflammation in the rats [77]. High levels of IL-1β and TNF-α recorded in this study may also be responsible for the activation of NF-κB and its translocation to the nucleus [85]. The anti-inflammatory effects of SYR and ASC were exerted as evidenced by a significant reduction in levels of TNF-α and IL-1β, and these could be due to their cytoprotective and antioxidant properties [55,86].

p53, a tumor suppressor protein, is a cell security protein that guards the cells against any cellular dysfunction via coordination of cell cycle function (arrest) and apoptosis [87,88]. Mdm2 on the other hand, is a target of Akt and an inhibitor of p53 protein and as such can control and regulate p53 signaling pathway [89]. In this study, the significant increase in lung p53 expression as a result of NDMA administration is an indication of lung damage in the rats. Oxidative stress that is marked by overproduction of free radicals and overwhelming of the endogenous antioxidant systems resulting from NDMA administration may be responsible for the damage, leading to the activation of p53 and subsequent activation of downstream pro-apoptotic players [90]. Lung mdm2 expression on the other hand was significantly lowered in the NDMA only animals despite the high mRNA expression of Akt. This might have occurred in the animals as a means to allow apoptosis to take place. This could have been through PTEN inhibition of mdm2 in the rats. The PI3K-Akt signaling pathway is also involved in the activation of cyclin D1, a major stakeholder in the progression of cell cycle. In this study, significantly low lung cyclin D1 expression was seen as a result of NDMA exposure. Since cyclin D1 is involved in the progression of G1 to S phase of cell cycle, the reduced cyclin D1 level recorded may have resulted to block cell proliferation and ensure cellular apoptosis. We propose that the mechanism that led to the reduced cyclin D1 level may be via p53 activation of p21, which in turn inhibited cyclin D1, and thus, block proliferation of damaged cells to favor apoptosis. Both Bax and Bcl-2 are involved in apoptosis. While the former is pro-apoptotic, the latter is anti-apoptotic, and they regulate apoptosis through control of mitochondrial function [91,92]. Overproduction of Bcl-2 stops mitochondrion pore formation, and so its inhibition potentiates cytochrome c release [91,93]. Inhibition of Bcl-2 and up-regulation of Bax leads to the formation of Bax homodimer, hence, the up-regulation of apoptosis [94]. From our findings, the simultaneous increase in Bax as well as Bax/Bcl-2 ratio and decrease in Bcl-2 by NDMA administration is an indication of p53-induced apoptosis, since both apoptotic players are coordinated by p53. Extruded cytochrome c from the mitochondrion interacts with apoptotic protease activating factor 1 (Apaf-1), which favors the activation of procaspase-9 [95,96]. Once activated, caspase-9 encourages the activation of downstream caspase-3 that is involved in executing apoptosis [97]. This explains the increase in caspase-3 level after NDMA administration observed in this study. The significant decrease in lung p53, Bax, caspase-3, Bax/Bcl-2 ratio and a concomitant increase in Bcl-2 and mdm2 levels by SYR and ASC acids are indications that both compounds possess anti-apoptotic properties, capable of stopping NDMA-induced apoptosis. In this study, the anti-apoptotic mechanism of both SYR and ASC is via mdm2-induced inhibition of p53, leading to Bcl-2 upregulation and inhibition of Bax and caspase-3.

Akt is involved in the regulation of cell survival through the inhibition of pro-apoptotic signals, including FoxO1, a transcriptional factor. FoxOs promotes target gene transcription, thereby stimulating cell cycle arrest and cell death [98]. Phosphorylation by Akt inactivates this transcription factor, leading to their cytoplasmic degradation and thus ensuring cell survival. Increased mRNA expression of lung FoxO1 seen in this study after NDMA administration and before treatments with SYR and ASC may be as a result of cellular response to apoptosis, which may have resulted to prevent the unwanted proliferation of damaged cells. This observation was further supported by the increased levels of other apoptotic markers including p53, Bax, caspase-3, and decreased level of Bcl-2 that were also observed in this study following NDMA administrations. Administration of ASC was the only treatment that was



effective in lowering the mRNA expression of FoxO1. This implies that the anti-apoptotic mechanism of ASC may be FoxO1 gene related, but may not be the case for SYR.

## 5. Conclusion

Findings from this study have revealed the participation of the PI3K-Akt-mTOR pathway in the etiology of NDMA-induced lung fibrosis. Both SYR and ASC investigated in this study have rich antioxidant, anti-inflammatory, and anti-apoptotic properties that were exerted via PTEN-mediated inhibition of PI3K-Akt-mTOR, NFκB, and mdm2-p53 signaling pathways. SYR had better therapeutic effect, and may join wortmannin and LY294002 in the league of drugs that are PI3K-Akt inhibitors.

## Funding

We received none.

## Declaration of interest

None to declare.

## CRediT authorship contribution statement

**Oluwatobi T. Somade:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Olubisi E. Adeyi:** Methodology, Investigation, Resources, Supervision, Project administration. **Babajide O. Ajayi:** Methodology, Investigation, Resources, Supervision, Project administration. **Osiro O. Asunde:** Methodology, Investigation, Resources, Supervision, Project administration. **Precious D. Iloh:** Methodology, Investigation, Resources, Project administration. **Adedayo A. Adesanya:** Methodology, Investigation, Resources, Project administration. **Olanrewaju I. Babalola:** Methodology, Investigation, Resources, Project administration. **Oluwaseyi T. Folurunsho:** Methodology, Investigation, Resources, Project administration. **Deborah A. Olakunle:** Methodology, Investigation, Resources, Project administration. **Opeyemi F. Lawal:** Methodology, Investigation, Resources, Project administration.

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