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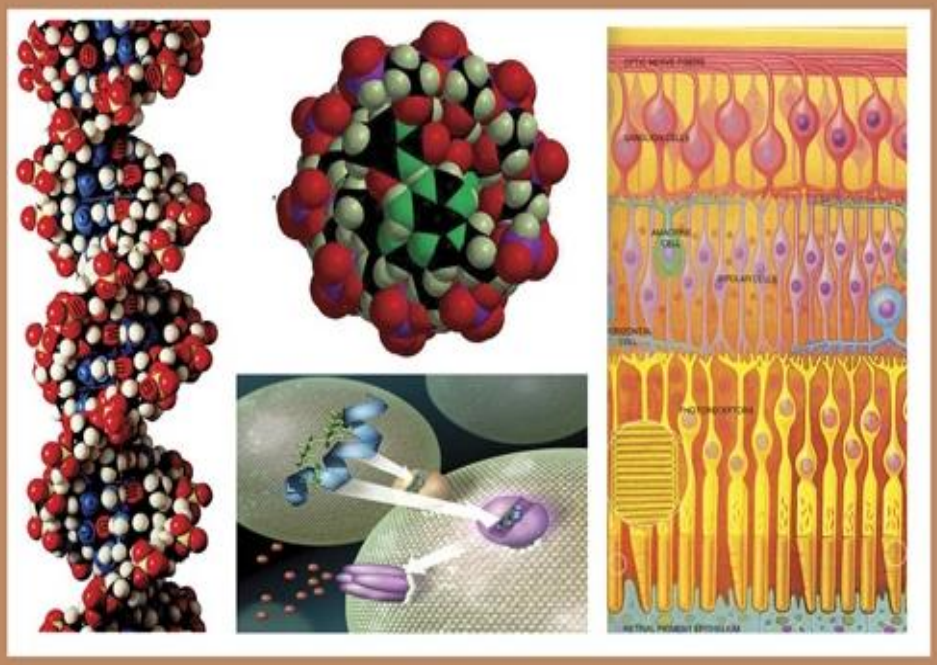

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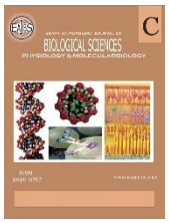

Assessment of Safety of Inhaled PMF Isolated from Camel Urine with Potential Activity against COVID-19

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ABSTRACT

PMF is a mixture of compounds extracted from lyophilized camel urine. It has many therapeutic effects including anticancer/antiviral activities. It has also a good safety profile. The aim of this study is to assess PMF safety in mice lung tissue after repeated inhalation. Adult male mice (N=15) were used. Clean surgical pads were used as cage bedding to avoid inhalation of wooden bedding. PMF aqueous solution, low and high doses were provided by whole-body inhalation exposure system, once daily for 3 successive days. At the end of the experiment, blood samples were collected, animals were sacrificed, and both lungs were taken for immunological and histology investigations. PMF inhalation in low and high doses showed normal levels of pro-inflammatory cytokines (TNF alpha and IL6) as well as normal antioxidants (GSH, SOD and catalase) in lung tissues. Whereas PMF inhalation in high doses led to an increasing level of MDA. The histological assessment also showed low or mild changes in both alveoli and bronchioles which are more obvious in the case of a high PMF dose. In conclusion, PMF inhalation either in high or low doses does not likely to induce an immunological inflammatory response in lung tissue. However, high doses may cause some histological changes.

INTRODUCTION

COVID-19 is a global pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). As of the end of June 2022, about 6.32 million cases had been reported, with four million deaths (Ciotti e*t al.,* 2019; Chakraborty and Maity 2020; Yang *et al.,* 2020; Roser *et al.,* 2021). Concerning, the use of oral or parenteral route in the management of COVID-19, most antiviral drugs demonstrated inconsistent results or limitations in clinical trials. The following is a brief explanation of representative drugs e.g., Remdesivir (RDV), which has many adverse effects including severe bradycardia (Touafchia *et al.* 2021), ECG changes (Bistrovic and Lucijanic 2021) anaphylaxis; liver and renal toxicity (Jorgensen, Kebriaei, and Dresser 2020). Lopinavir/ritonavir (LPV/r), a meta-analysis concluded no significant advantage of LPV/r in alleviating symptoms of COVID-19 (Alhumaid *et al.* 2020). Explanation for the ineffectiveness of the drug was the subject of several published studies which highlight its high protein binding and poor access to lung tissues(Ali *et al.,* 2021). However, a Pharmacokinetic (PK) study in critically ill COVID-19 patients who received the recommended dose of favipiravir (FPV) demonstrated a low trough level (1 µg/mL) (Mobinizadeh and Arab-Zozani 2021). It has low potency against SARS-COV-2, EC_{50} is 61.88 µM (Wang *et al.,* 2020). The lung-to-tissue level of FPV was estimated to be about 50 % of that in the blood. These PK data suggest moderate drug access to lung tissues (Irie *et al.,* 2020). The drug has some adverse effects, including a rise in serum uric acid, liver enzyme diarrhea nausea, vomiting, and tachycardia (Kaur *et al.,* 2020). Hydroxychloroquine (HCQ) absorption of HCQ is extensively variable (~70%; range: 25 to 100%). HCQ has a narrow therapeutic range (Furst 1996), Ali *et al.*,2021, suggested that HCQ is not likely to provide a potent antiviral effect in severe cases of COVID-19. Another limitation of HCQ is potential QT prolongation and ventricular arrhythmia and there has been no dose-response relationship study to accurately predict the association of HCQ drug level with this cardiac toxicity (Horby *et al.,* 2020; Javelot *et al.,* 2020; Juurlink 2020).

The SARS-CoV-2 virus induces severe acute respiratory syndrome (SARS) that mainly impacts the airways. It was considered complicated viral pneumonia which may lead to death (Hasöksüz, Kilic, and Saraç 2020).

Antiviral/anti-inflammatory drugs administered via the pulmonary route represent an effective method of treating the disease. Such a method has proved successful when treating other conditions affecting the lungs, possibly due to its quick action, low metabolism and decreased systemic adverse effects (Labiris and Dolovich 2003; Albariqi et al. 2021; Tai *et al.,* 2021)

Some reviews provided recent studies of inhaled antiviral drugs with promising activity against the novel virus or lung inflammation, these include pre-clinical formulation, safety and efficacy in an animal model and a few clinical trials(Alrashedi *et al.,* 2021; Saha, Quiñones-Mateu, and Das 2022)

Natural products are an important source of novel drug therapies with diverse biological activities and unique chemical structures. Camel urine is known as a traditional treatment for many diseases (Alkhamees and Alsanad 2017; Gole and Hamido 2020)

PMF is a mixture of compounds extracted by alcohol from PM 701 (lyophilized camel urine). It was demonstrated to selectively inhibit certain cancer cells (Ali *et al.,* 2011; Alebie, Yohannes, and Worku 2017)

Prospects of utilizing nano-formulation were recently explored to optimize its efficacy in cancer therapy(Ahmed et al. 2015). PMF was proved to be a promising virucide when tested on the Vesicular stomatitis virus (VSV), a virus model which was completely inhibited 75 minutes post-treatment with PMF(Al-Attas *et al.,* 2015) PMF also had an in vitro antiviral

activity against H1N1. (Al Attas *et al.,* 2019) Moreover, a quantitative virucidal suspension test was performed using the Transmissible gastroenteritis coronavirus (TGEV) virus as a model for MERS-CoV. The results specified that when applying PMF at a concentration of 20.34% inactivated the virus by 99.5% after 60 min, while 99.90% after 240 min(Al Attas et al. 2019).

The present study aimed to study the safety of PMF on mice lung tissue if given via inhalation route.

MATERIALS AND METHODS

The study was approved by the ethical approval committee at King Fahd Medical Research Center (KFMRC 2.3.2005). Adult male SWISS mice (N=15) were purchased from the animal house (KFMRC). The average body weight at the experiment time was (20-25 gm). Mice were housed in (transparent polycarbonate cages, dimensions in cm about 15 W x 20 LX 46 H), with a maximum of 5 mice/cage. Lab conditions: temp 22 ±1, relative humidity of 50 ±10%, 12-h light-dark cycle, constant air change, standard diet and tap water ad libitum. Animals were transferred to a clean inhalation chamber (25 X 20 x 40 cm) just before inhalation and transferred back to their cages after inhalation. The animals were administered PMF aerosol Via Whole-body inhalation(Cidem *et al.,* 2020) for 3 min once daily (9-10 Am), for three successive days. See the experimental design in Table. The aerosol of the PMF solution was generated by a portable Nebulizer machine (CompAir® Compressor Tabletop Nebulizer System Model: NE-C801 (Fig. 1). The solution of PMF (2 ml) or Saline was placed in the nebulizer reservoir and the mouthpiece was placed in the inhalation chambers. The appropriate weight of PMF powder was dissolved in 2 ml of sterile saline at specified (low concentration of the PMF, 0.01 gm per mouse and high concentration of the PMF, 0.1 gm per mouse. These solutions were prepared just before the ore administration.

Fig. 1: The used tabletop Nebulizer System

Table 1: Experimental Design

Group	The procedure: inhalation time 3 min. once daily, three successive days, Whole-body inhalation
Control (n=3	The normal group, receive saline (2 ml).
G2 n=5	A low dose of PMF 0.01 gm per mouse, 2 ml in normal saline,
G3 n=5	high dose PMF 0.1 gm per mouse, 2 ml in normal saline,

Collection of Blood Samples and Lung Tissues:

On day 4, mice received IV pentobarbital 50mg/kg. Blood samples were collected, (Eppendorf tubes (0.5 mL); using capillary tubes (Micro Hematocrit Capillaries, Mucaps) from the retro-orbital intravenous plexus. The samples were centrifuged (3000 rpm, 15 minutes), and serum was separated and kept at – 80 °C until examination time within 7 days.

After blood collection, the animals were sacrificed by decapitation. Both left and right lungs were removed weighed and cut into thin slices. Some lung slices were fixed in 10% neutral buffered formalin for histological assessment and the remaining were used to prepare tissue homogenate.

Preparation of Tissue Homogenate:

100 mg of the frozen lung was weighed; 1 ml of ice-cooled PBS (pH 7.4) was added, and homogenization was done using a polytron homogenizer (PT 3100) (five cycles of 10 s at 3000 rpm). The homogenate was centrifuged at 3000 rpm for 20 min at – 8 °C (using a HERMLE centrifuge, Germany). The supernatant was collected into a pre-chilled Eppendorf tube and stored at -80 °C till assayed.

Assessment of Certain Immunological and Biochemical Markers:

The following parameters were determined using the ELISA, and the procedure and calibration were according to the manufacturer's instructions. Reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT); malondialdehyde (MDA), tumour necrosis factor-alpha (TNF-α) and Interleukin -6 (IL-6).

Statistical Analysis:

Data were expressed as mean +/- standard error of the mean. Analysis was made by IBM SPSS Statistics for Windows, version 23 (IBM SPSS, IBM Corp., Armonk, N.Y., USA). Shapiro – Wilk test was used to evaluate normal data distribution. A one-way ANOVA test followed by Tukey's test, assuming groups' equal variance, was utilized to calculate significance. *P*-values of <0.05 were considered statistically significant.

RESULTS

Mice's total body and lung weights after inhalation of PMF are shown in figure 2. The total lung weight/ body weight (at the end of the experiment) showed a trend to increase in relative lung weight due to inhalation of PMF which is slightly higher with a high PMF dose; However, these changes were statistically insignificant p = 0.22.

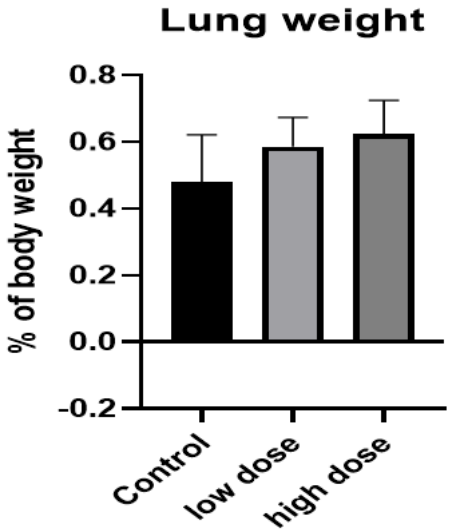

Fig. 2: Increase in mean % of both lungs / total body weight of mice after inhalation of low and high doses of PMF for 3 days**.**

Oxidative Stress Markers, Pro-Inflammatory Cytokines, And Antioxidants In Mice Lung Tissue Homogenate:

Table 2 and Figure 3 showed that in mice lung tissue homogenate, the mean levels of MDA were significantly higher due to inhalation of high PMF dose versus the control (*P* <0.0001)

Meanwhile, there were insignificant changes in other oxidative stress and pro-inflammatory markers between different studied groups.

Table 2 Biochemical and immunological parameters in mice lung tissue homogenate after repeated inhalation of high and low doses of PMF for 3 days.

Parameters	NC group	High dose group	Low dose group
GSH (ng/ml)	16.40±1.65	20.10±2.22	16.0±3.11
SOD (u/ml)	181.00±4.58	177.25±10.44	176.80±6.54
CAT (Mu/L)	113.67±4.16	115.50±3.87	117.40±4.34
MDA (nmol/ml)	0.33±0.05	0.90±0.12*	0.37±0.07#
TNF-α (pg/ml)	12.60±0.96	12.08±1.36	14.36±1.73
IL-6 (pg/ml)	5.12±0.33	4.55±0.64	4.76±0.98
*: high dose group versus the control group (p = 0.001). #: low dose group versus high dose (P = 0.001)			

Data were expressed as mean± standard deviation. Comparison between groups was made by one-way ANOVA followed by Tukey's test.

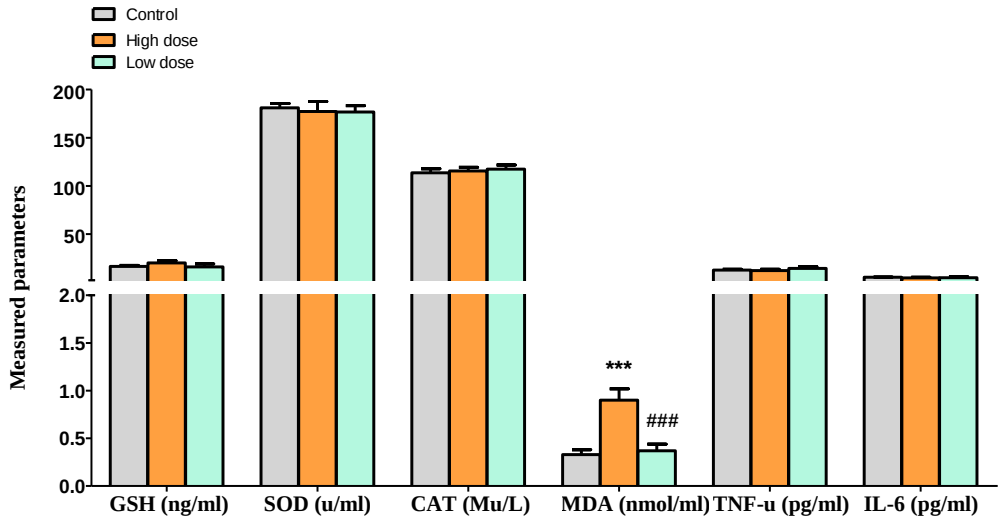

Fig. 3: Biochemical and immunological parameters in mice lung tissue homogenate after repeated inhalation of high and low doses of PMF for 3 days.

Data were expressed as mean+/= standard deviation. Comparison between groups was made by One-way ANOVA followed by Tukey's test. *: high dose group versus the control group (p = 0.001). #: low dose group versus high dose (P = 0.001).

Histological Findings:

Histological examination of H&E-stained sections (Fig.4) showed that in the low-dose PMF group, the lung alveoli appeared patent; bronchiole epithelium was intact. Only a few scattered closed alveoli were observed. Inhalation of high PMF dose, on the other hand, resulted in marked alteration of most alveoli, they were closed by inflammatory cells with increased interalveolar inflammatory mononuclear cell infiltrate. Some bronchioles showed mucous secretion.

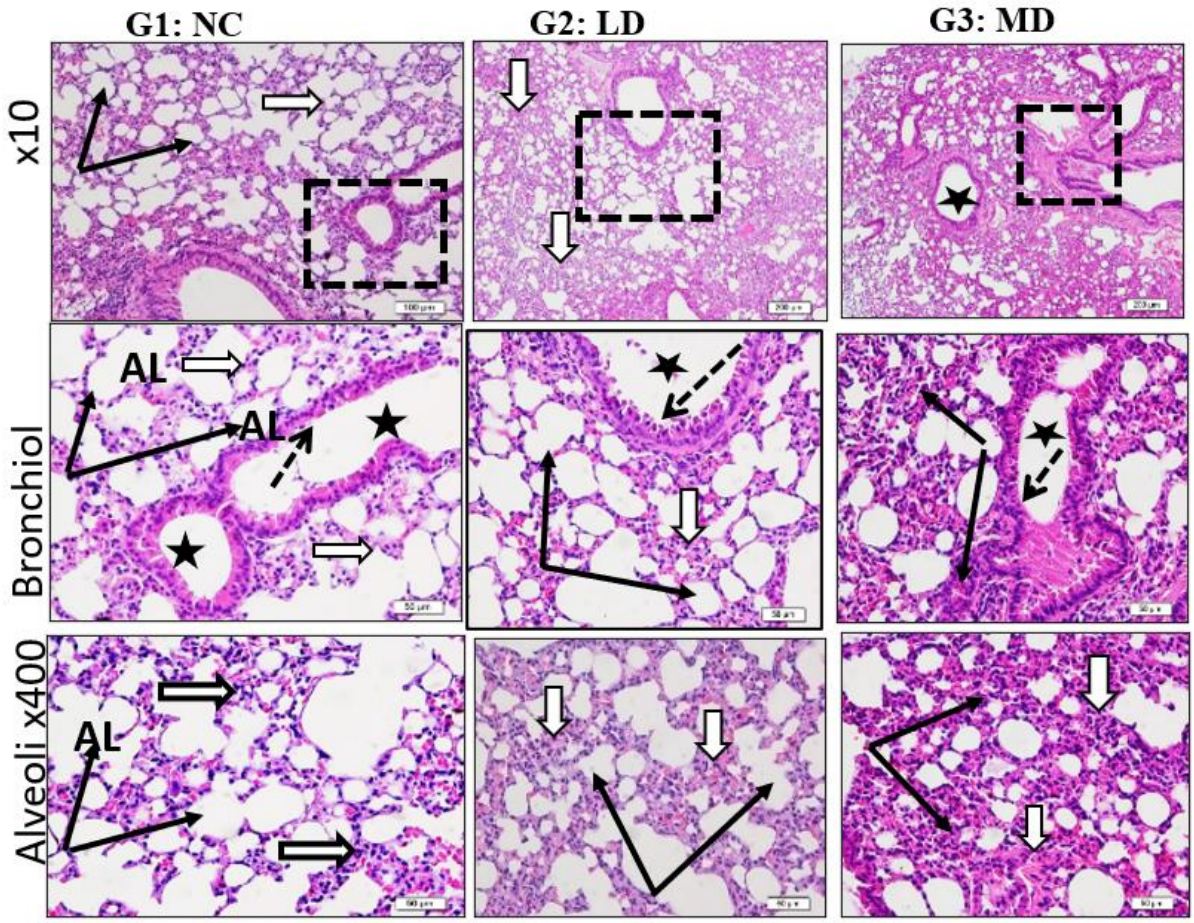

Fig (4): Paraffin sections of mice lung tissues stained by H&E to show:
G1: Control low (x100) and high powers (x400) to show both normal patent alveoli (black arrows, AL) with thin inter-alveolar septa containing few connective tissue cells (white arrows). Numerous sections of the bronchiolar tree (dotted square) could be seen lined by normal columnar epithelium (dotted black arrow) and have empty lumina free of mucous or cellular deposits (star).
G2: Low dose of PMF Showing no alteration in alveolar patency (black arrows) with only a very mild increase in septal thickness (white arrows). Bronchiole epithelial lining (dotted arrows) with a patent lumen(star) and other normal features.
G4: High dose of PMF (showing marked alteration with many alveoli, closed by inflammatory cells (black arrows). Increased interalveolar inflammatory cells (white arrow) are observed. Some Bronchioles showed mucous secretion (star).

DISCUSSION

The human lung is a highly perfused tissue with a large surface area, which leads to high bioavailability with rapid onset of action of inhaled medications. These features make the pulmonary delivery system an optimal route for antiviral and immunomodulators indicated for the management of COVID-19. Optimized lung delivery systems, allow drugs to selectively target lung tissues (enhanced efficacy) with minimal systemic toxicity (minimize toxicity), ensure a fast effect, and decrease variability. These favourable features could effectively optimize the pharmacotherapy of COVID-19 (Eedara *et al.*, 2021). In the present study, PMF was administered by whole-body inhalation to mice as a preliminary safety investigation.

Several types of cells are involved in lung inflammation, including the epithelial cells that line the airways and alveoli and the

immune cells in interalveolar tissue. These cells also secrete cytokines and chemokines that serve as inflammatory mediators that promote inflammatory reactions and fibrosis (Moldoveanu *et al.,* 2009). During the acute phase of inflammation, neutrophils rapidly migrate to the lung as first responders, producing reactive oxygen species(Potey *et al.,* 2019). Several investigations have shown that TNF- α plays a key role in the inflammatory process. It controls critical physiological operations (such as cell differentiation, and apoptosis) as well as a wide range of reactions to stress and damage (Zelová and Hošek 2013). Overproduction of TNF- α is critical in the stimulation of inflammatory genes, cell death, endothelial up-regulation, and the recruitment and activation of immune cells during inflammation. It has also been identified as a significant modulator of systemic progression and tissue destruction in severe illness (Shen and Pervaiz 2006). In the present study, we demonstrated no significant increase in lung tissue expression of TNF- α and Il-6 in response to the inhaled PMF either low or high dose but the elevation of only one marker for oxidative stress MDA with high dose.

The production of epithelial cytokinesis is related to acute lung inflammatory response as well as the activation of the signaling cascade that leads to apoptosis (programmed cell death). Tumor Necrosis Factor (TNF)-mediated activation of epithelial proinflammatory signaling cascades (Manicone 2009) (Varfolomeev and Ashkenazi 2004). The following cascade has been proposed to explain TNF-function a's in lung inflammation that results in tissue damage: TNF-> endothelial/epithelial TJ modification -> polymorphonuclear (PMN) infiltration -> increased pro-inflammatory mediator release -> organ damage (Mazzon and Cuzzocrea 2007). In the present study, histological changes are more prominent with high doses of PMF, suggesting dose-dependent direct toxicity.

The PMF compound is a crude mixture, likely containing compounds (e.g. benzoic acid, and hippuric acid) at high concentrations deposition of insoluble particles likely to occur leading to chemical pneumonia (Teramoto 2020). A chemical-induced respiratory injury produced by inhaled chemical agents is mostly determined by the kind and quantity of the substance breathed (Andujar and Nemery 2009). For example inhalation of diesel exhaust particulate caused a dose-dependent elevation in TNF-α expression in lung tissues in a mouse model (Kumar *et al.,* 2017). The immediate and chronic effects of breathing were established in another work. Where mixed macrophage phenotypes (M1/M2) and T helper cells activation of both TH1 and TH2 subtypes were implicated in an analysis of immunoregulatory and pro-inflammatory cytokines in serum and airways. The pro-fibrotic cytokine was found to be overexpressed in the airways, 24 hours after exposure and remained so at later time points (14 and 28 days). 14 days after exposure, histopathological examination revealed substantial collagen deposition.(Wigenstam *et al.,* 2016).

The present study demonstrated features of increased oxidative stress after exposure to a high dose of PMF. In airway illnesses, oxidative stress plays a crucial part in harmful and inflammatory responses. (Park, Kim, and Lee 2009; Sethi, Dharwal, and Naura 2019; Hecker 2018). Oxygen is required for complicated biological life since it is required for cellular metabolism and energy generation. Highly reactive intermediates, or reactive oxygen species (ROS), like superoxide (O_2) and hydrogen peroxide (H_2O_2), are produced during the breakdown of oxygen and perform critical physiological intracellular functions. Aerobic metabolism produces free radicals; 2–3 per cent of the oxygen used by a cell is transformed into free radicals (Holmström and Finkel 2014).

Various cell types in the lung create oxidants, including inflammatory (neutrophils and macrophages), fibroblasts, endothelial, and epithelial cells. ROS-producing enzymes such as NADPH oxidase, nitric oxide synthase, lipoxygenase, and

xanthine oxidase are expressed in these cells. Because oxidants can work indiscriminately to change adjacent biomolecules, a large arsenal of cellular antioxidant defences has developed to assist restore oxidative equilibrium, guard against damage, and preserve cellular homeostasis. Enzymatic (superoxide dismutase, catalases, peroxiredoxins, glutathione systems) and nonenzymatic (vitamins and amino acids) antioxidant sources exist naturally or through food consumption and have either direct antioxidant properties or serve as progenitors or co-factors for enzymatic antioxidants.

Endogenous oxidant-antioxidant systems play a key role in cell homeostasis and environmental stress response. ROS plays a key part in physiological signaling events that affect a variety of cellular processes, including DNA integrity, cellular senescence, apoptosis, and ECM remodeling (Torrens-Mas *et al.,* 2020; Andrew 2001). During normal (physiological) injury-repair reactions, all these cellular mechanisms are critical.

Although there are limitations in this research represented in the short time of the experiment. The research is unique as the first experiment to test the interaction of compounds from camel urine that are potentially effective against viruses by inhalation

Conclusion:

In this research, a simple procedure was used to assess the safety of inhalation of an aqueous solution of a mixture of compounds extracted from camel urine. It was found that high doses cause oxidative stress and histological changes likely of chemical nature. In small doses of PMF, minimal changes were documented.

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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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