Original Research Article

Evaluating the Antioxidant Potential of Lemon grass Extract in Alleviating Paraquat-Induced Oxidative Stress in Sheep Red Blood Cells: An In-Vitro Investigation with Phytochemical Analysis.

ABSTRACT

Aims: Oxidative stress, a key player in various diseases, notably affects cellular health. Erythrocytes are particularly vulnerable to free radical damage, making them prime targets for harm. Lemon grass possesses strong antioxidant capabilities attributed to its abundant polyphenolic acid and flavonoid content, making it highly notable. Our study delved into lemon grass' antioxidant expertise against oxidative stress from Paraquat in sheep red blood cells (RBCs), including a comprehensive phytochemical analysis of lemon grass.

Methodology: Lemongrass leaf extract was prepared by drying and grinding leaves, followed by Soxhlet extraction with acetone. Phytochemical analysis was done to reveal the presence of alkaloids, saponins, flavonoids, tannins, phenols, and acids. A 10% RBC suspension was prepared from sheep blood. Oxidative stress was induced in RBCs using Paraquat, Ascorbic acid, and the extract. Antioxidant analysis included protein concentration via the Lowry method, TBARS estimation for lipid peroxidation, GSH estimation, and SOD activity assay. Statistical analysis was performed using GraphPad Prism, with significance set at $p<0.05$.

Results: The phytochemical analysis revealed the presence of flavonoids, terpenoids, saponin, and tannin in lemongrass, while no acids or alkaloids were detected in the plant.In Group II exposed to Paraquat toxicity, total protein decreased significantly compared to the control group (10.24±0.53 to 6.3±0.49). TBARS activity notably increased in Paraquat-treated group (629.7±2.85) compared to control (398.8±3.51), while Lemongrass and ascorbic acid treatments showed significant decreases (513.05±4.54 and 504.01±1.97, respectively). SOD and GSH levels decreased significantly in Paraquat-treated group (6.08±0.4 and 80.86±4.9 µg GSH/mg of protein) compared to control (11.13±1.1 and 109.81±8.6, respectively), but Lemongrass treatment increased levels (14.12±2.0 and 114.8±4.5 µg GSH/mg of protein). Lemongrass demonstrated pronounced protective effects against oxidative stress, normalizing SOD levels and increasing GSH levels, comparable to ascorbic acid treatment.

Conclusion: Our study successfully induced oxidative stress in RBCs with Paraquat, presenting avenues for further exploration in understanding cellular responses. Additionally, our research highlights Lemongrass's significant preventive impact (100µg), possibly attributed to its bioactive phytochemicals, including flavonoids, phenols, saponins, and tannins. This underscores Lemongrass's potential in countering oxidative damage induced by Paraquat in RBCs.

Keywords: Lemon grass, Paraquat, Oxidative stress, Flavanoids, RBC, Ascorbic acid

1. INTRODUCTION

Oxidative stress arises as a physiological condition when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize and eliminate these harmful molecules[1]. Reactive oxygen species consist of highly reactive oxygen-based molecules, including free radicals (molecules with unpaired electrons) and specific peroxides. They naturally occur as byproducts of various cellular processes, such as metabolism, immune responses, and energy production [2]. However, if the generation of ROS exceeds the body's protective antioxidant mechanisms, it can result in the onset of oxidative stress. This condition holds the potential to cause damage to cellular components like proteins, lipids, and DNA, thus contributing to the development of various diseases[3].

Paraquat is a widely used herbicide introduced in the 1960s, known for its efficient weed-killing abilities across various plant species. However, its notoriety arises from the risks it poses to human, animal and environmental health due to its potential toxicity and oxidative stress induction [4]. Upon contact with cells or organisms, paraquat triggers the production of reactive oxygen species (ROS), disrupting the balance between oxidative stress and protective antioxidant mechanisms. This process, known as redox cycling, is exacerbated by ROS disturbing organelle functions, causing harm and hindering protective systems[5]. Within cells, paraquat engages in redox reactions, utilizing enzymes like NADPH and CYPs reductase. This leads to the generation of superoxide radicals (O_2^-) through electron transfer. These radicals can convert to hydrogen peroxide (H_2O_2), another form of ROS, intensifying oxidative stress and damaging cellular structures [6]. ROS overproduction harms cellular components like lipids, proteins, and DNA, disrupting cell function and stability. Paraquat-induced oxidative stress affects mitochondria, essential for cellular energy production. This disturbance triggers further ROS generation, perpetuating oxidative stress. Oxidative stress activates cellular signalling pathways related to stress responses, inflammation and cell survival, influencing the cellular response to paraquat-induced stress [7].

Paraquat's highest toxicity is observed in the respiratory system, particularly targeting the lungs. Regardless of the exposure route, the lungs selectively accumulate paraquat, disrupting lung cellular structure and causing pulmonary fibrosis. This results in respiratory failure and symptoms like chest tightness and shortness of breath. Lung inflammation, swelling and pleural fluid accumulation contribute to fatal outcomes [8]. Paraquat rapidly spreads throughout the body, with the kidneys accumulating the most. This accumulation increases the risk of severe nephrotoxicity, leading to acute kidney injury, especially in the proximal tubules. Within 24 hours, paraquat induces degeneration and necrosis in mouse renal tubules [9]. In summary, paraquat disrupts the redox cycle, inhibits antioxidants and generates potent oxidants causing cellular damage. Its impact varies across target organs, notably affecting the lungs, brain, liver, and kidneys, intensifying oxidative stress and amplifying cellular harm [6].

Cymbopogon citratus, commonly referred to as lemon grass, is a tropical perennial herb belonging to the Poaceae family, which comprises true grasses. This aromatic tall grass has rhizomes and densely tufted fibrous roots [10]. It has been traditionally used in folk medicine to address issues like nervous and gastrointestinal disturbances, fever and hypertension. Lemon grass finds culinary use as a flavor enhancer in various dishes. Furthermore, its infusion as lemon grass tea offers a revitalizing beverage option. The plant is valued for its essential oil, primarily due to its constituents, citronellal and citral[11]. These compounds serve essential roles in detoxification and exhibit anticancer properties by stimulating glutathione-S-transferase (GST) activity [12]. The components α-citral (geranial) and β-citral (neral) demonstrate antibacterial effects, effectively inhibiting the growth of both

Gram-positive and Gram-negative bacteria[13].Lemon grass has also been found to contain various phytochemicals, including flavonoids, alkaloids, volatile and non-volatile terpenoids, carotenoids and tannins [14].

2. MATERIAL AND METHODS

2.1 Leaf extract preparation:

Lemongrass leaves were gathered and naturally dried away from direct sunlight. Subsequently, they were finely ground into powder and stored securely in an airtight container. For the compound extraction process, 10 grams of this powder was mixed with 100 ml of acetone, initiating a 48-hour extraction within a Soxhlet apparatus. The solution was then filtered using Whatman Filter Paper No. 1 and is subjected for evaporation. The resultant extract, obtained after this procedure, was dissolved in DMSO at a concentration of 100 mg/ml. (Figure-1)

2.2 Phytochemicals analysis:

The qualitative phytochemical analysis of the lemon grass extract was conducted using the methods outlined by Claustra [15]and Harborne [16].

2.2.1Test for Alkaloids: To conduct the Alkaloid Test, 2ml of HCl added to 2ml of the extract. Following this, a few drops of Mayer's reagent were carefully incorporated. The presence of alkaloids is indicated by the emergence of a greenish coloration.

2.2.2 Saponin Test: To test for saponins, 2 ml of distilled water was mixed with 2 ml of the oil extract. The mixture was vigorously shaken for a duration of 15 minutes. The presence of saponins is indicated by the formation of foam.

2.2.3 Flavonoid Test: For the detection of flavonoids, 5 ml of diluted NH_3 solution was combined with 1 ml of the oil extract. Subsequently, concentrated sulphuric acid was added to the mixture. The presence of flavonoids is signalled by the appearance of a yellow coloration.

2.2.4 Tannin Test: A test for tannins involved adding 2 ml of 5% ferric chloride to 1 ml of the oil extract. The presence of tannins is indicated by the development of a greenish-black coloration.

2.2.5 Phenol Test: To determine the presence of phenols, 2 ml of distilled water along with a few drops of 10% ferric chloride were mixed with 1 ml of the oil extract. The presence of phenols is indicated by the appearance of a green coloration.

2.2.6 Acid Test: To examine for acids, 1 ml of oil extract was treated with a solution of sodium bicarbonate. The occurrence of effervescence indicates the presence of acids.

2.3 Preparation of 10% RBC suspension:

A volume of two millilitres of blood is obtained from the jugular vein of the sheep and carefully transferred into an EDTA vial. Following this, the collected blood is subjected to centrifugation at 1100 rpm for a duration of 12 minutes, maintaining a temperature of 5°C. The resulting plasma is then carefully washed with PBS and this washing process being repeated three to four times until a clear supernatant is achieved. In order to establish a 10% RBC solution, a mixture containing 200 µl of RBC pellet and 1800 µl of cold PBS is meticulously prepared. This solution is subsequently stored at a temperature of -20°C, ensuring its preservation for future utilization. (Figure-1)

Fig.1: Pictorial representation of extraction and RBC suspension preparation.

Table-1 Experimental Design

Group	Treatment
I	RBC control (no treatment)
II	RBC + Paraquat (50µM)
III	RBC + Paraquat (50µM) +Ascorbic acid (100µg)
IV	RBC + Paraquat (50µM) + Lemongrass (100µg)

2.4 Induction of oxidative stress:

Distinct sets were created from the 10% RBC suspension and subjected to different concentrations of Paraquat, Ascorbic acid and Lemon grass extract. The resulting mixtures were then incubated on an orbital shaker for 24 hours according to the experimental design.

2.5 Antioxidant analysis:

2.5.1 Estimation of protein concentration:

Protein concentration was determined employing the Lowry method. In this approach, proteins react with copper ions in an alkaline solution, generating a color complex. The color intensity is directly proportional to protein concentration. The absorbance of the complex was measured at around 750 nm using a spectrophotometer. The Lowry method is esteemed for its reliability and sensitivity in protein estimation [17].

2.5.2 Estimation of TBARS:

The assessment of tissue lipid peroxidation in pancreatic tissue followed a previously documented method [18]. In summary, approximately 100 mg of tissue was homogenized using 500 µl of PBS. Subsequently, the entire homogenate (100 µl) was

combined with 100 µl of 8.1% SDS, 750 µl of 20% acetic acid, 750 µl of 0.8% TBA and 300 µl of ultrapure water. This mixture was then subjected to heating in a water bath at 95°C for 60 minutes, followed by cooling and subsequent centrifugation at 10,000 rpm for 10 minutes. The resulting supernatant was measured at 532 nm for quantification purposes.

2.5.3 Estimation of GSH:

The homogenate obtained from the previous step for the MDA assay was subjected to centrifugation at 6,000 rpm for 5 minutes. Following this, 50 µl of the sample was combined with 120 µl of Ellman's reagent and 100 µl of GSH buffer, as outlined [19]. The resulting mixture was then allowed to incubate in darkness for a duration of 10 minutes, after which the absorbance was measured at 412 nm.

2.5.4 Estimation of SOD:

The reaction entails the production of superoxide through pyrogallol autooxidation, coupled with the suppression of superoxide-dependent reduction of the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to formazan. This reaction is measured at 570 nm [20].

2.6 Statistics:

The experimental results were expressed as the mean ± standard error (SE) values. Statistical analysis was carried out utilizing Graph Pad Prism Software version 5.0. This encompassed a one-way analysis of variance, followed by Tukey's multiple comparison test. The significance of the observations was established at a significance level of $p<0.05$.

3. RESULTS AND DISCUSSION

Table 2 displays the identified phytochemicals in the lemon grass extract. The findings indicate the presence of flavonoids, terpenoids, saponin and tannin whereas no acids or alkaloids were found to be present in lemon grass.

Table-2 Phytochemical analysis

Name of Phytochemical	Result
Alkaloid	-
Saponin	+
Phenol	+
Flavanoids	+
Tannin	+
Acids	-

In the second experimental group (Group II) exposed to toxicity, the total protein content decreased to 6.3±0.49, notably lower than the control group value of 10.24±0.53. Notably, the examined groups, Groups III and IV, showed significant reductions in total protein levels as well, measuring 6.78±0.59 and 6.9±0.5, respectively.

In Group 2 exposed to Paraquat treatment, the Thiobarbituric Acid Reactive Substances (TBARS) activity (measured in nM/mg protein) notably increased to 629.7±2.85. This surge in TBARS activity was significant ($p<0.001$) when compared to the control group (398.8±3.51), comprised of RBCs. Conversely, the treatment with Lemongrass (Group IV)

and ascorbic acid (Group III) resulted in significant decreases in TBARS levels ($p<0.001$ and $p<0.01$, respectively), with values measuring 513.05±4.54 and 504.01±1.97. These results highlight the potential protective effects of Lemongrass and ascorbic acid in mitigating oxidative stress-induced damage.

The activity of Superoxide Dismutase (SOD) (measured in U/mg protein) and Glutathione (GSH) (measured in µg/mg protein) experienced a noteworthy reduction ($p<0.01$) in Group 2, treated with Paraquat, compared to the control group (Group 1 - RBCs). Specifically, the GSH levels in red blood cells (RBCs) treated with 50µM Paraquat were measured at 80.86±4.9 µg GSH/mg of protein, in contrast to the GSH level in the RBC group (109.81±8.6). Treatment with Lemongrass resulted in an increase in GSH levels to 114.8±4.5 µg GSH/mg of protein. Notably, Group III, treated with ascorbic acid, also exhibited GSH levels at 120.2±5.6 µg GSH/mg of protein, without significant differences.

The SOD levels were recorded as 11.13±1.1 SOD units/mg protein in the control group and a decrease to 6.08±0.4 SOD units/mg protein was observed in the toxic group (Group II) treated with Paraquat. In contrast, treatment with Lemongrass significantly elevated SOD levels to 14.12±2.0 SOD units/mg protein, indicating a pronounced protective effect. The plant extract exhibited substantial normalization of SOD levels, suggesting its potential protective properties. Interestingly, no substantial differences were found in values between Groups IV and III, with SOD values of 15.30±1.2 (Graphical representation are presented in Fig 2).

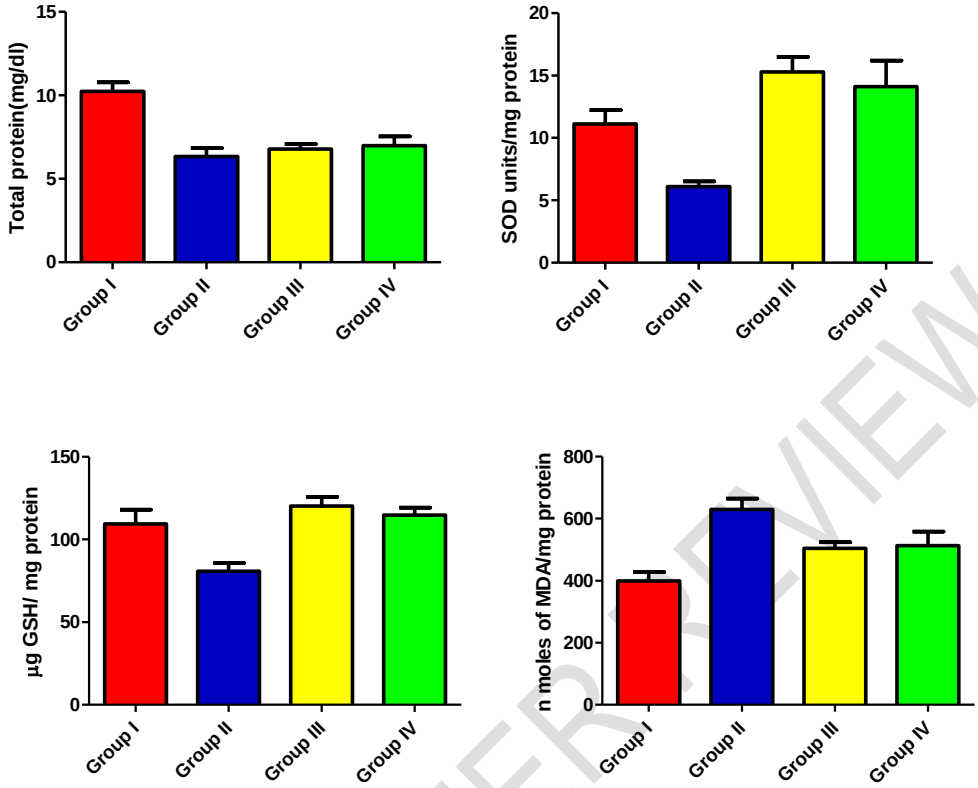

Fig.2: Graphical representation of Total protein, SOD, GSH and TBARS.

DISCUSSION:

Oxidative stress, as extensively discussed in the scientific literature, emerges when there is a significant imbalance between the production of reactive oxygen species (ROS) and the defensive mechanisms designed to counteract their detrimental impacts. This imbalance disrupts the intricate equilibrium between pro-oxidant and antioxidant factors, tipping the scale in favor of pro-oxidants. As a result, the potential for cellular damage becomes a distinct possibility [21]. In this context, the deliberate induction of oxidative stress within red blood cells (RBCs) using Paraquat as a stress-inducing agent has yielded valuable insights into the intricate mechanisms governing cellular responses to elevated levels of reactive oxygen species (ROS). Our study successfully replicated the widely acknowledged phenomenon of oxidative stress in RBCs, thereby shedding light on the complex cellular dynamics influenced by this specific stressor [22]. Upon the application of Paraquat to induce oxidative stress, a sequential cascade of cellular processes indicative of oxidative injury unfolded. The observation of a pronounced increase in the generation of reactive oxygen species (ROS) within the confines of the RBCs not only verified Paraquat's capability to initiate the production of free radicals but also provided quantification of the extent of this effect [23]. Subsequent to this accumulation of ROS, the consequential occurrence of lipid peroxidation in the cellular membrane was substantiated by the results. This underscores the disruptive potential of accumulated ROS within RBCs, as previously substantiated by established scientific literature[24]. The findings of our study further contribute to the comprehension of the intricate interplay between oxidative stress and

cellular responses, emphasizing the importance of safeguarding cellular components against the destructive consequences of ROS accumulation.

Lemongrass (Cymbopogon citratus) boasts diverse therapeutic qualities due to its abundance of phytochemicals. Notably, it contains polyphenols like flavonoids (e.g., quercetin, rutin) and phenolic acids (e.g., caffeic acid), contributing potent antioxidant properties for combating oxidative stress[25]. Lemongrass extracts, rich in flavonoids, also display free radical scavenging potential, reducing cellular damage caused by reactive oxygen species [26]. Additionally, lemongrass holds saponins, with their distinct structure facilitating interactions with both water-soluble and lipid-soluble molecules. This imparts various beneficial effects, including anti-inflammatory, antioxidant, immunomodulatory, and anticancer activities [27], enhancing lemongrass's potential as a natural source of versatile bioactives. It also contains tannins, recognized for their interactions with biomolecules and antioxidant qualities. Their role in scavenging free radicals aligns with lemongrass's potential for promoting well-being [28], with interactions influencing bioavailability and physiological effects.The rich assortment of bioactives in lemongrass underscores its therapeutic potential, necessitating further study to uncover synergistic effects and clinical applications, along with a comprehensive understanding of their interactions in cellular pathways for promoting health.

The accumulation of MDA, resulting from lipid degradation, is a reliable indicator of heightened lipid peroxidation [29]. This concurs with the decrease in intracellular glutathione levels and subsequent reduction in SOD activity observed during the stress period[30]. Elevated MDA levels effectively signify the presence of oxidative stress. The results notably revealed a marked MDA increase in the Paraquat group, followed by reductions in both the ascorbic acid and lemon grass groups[31, 32].

Confronted by the oxidative challenge from Paraquat, red blood cells (RBCs) boosted their innate antioxidant defenses. Enzymes like superoxide dismutase (SOD) and glutathione peroxidase heightened their activity to effectively counter reactive oxygen species (ROS). This responsive adaptation aligns with cellular strategies to combat oxidative stress damage. However, maintaining a delicate balance between ROS production and initiating antioxidant defenses remains crucial. This balance prevents overwhelming defense mechanisms and subsequent harmful cellular outcomes [33].

Lemongrass extract, rich in phyto constituents, exhibits active oxidative metabolism generating ROS [12]. The extract is also abundant in antioxidant enzymes, including GSH—an essential element in neutralizing free radicals and maintaining cellular integrity. GSH swiftly contributes hydrogen atoms to neutralize radicals, playing a pivotal role in cellular antioxidant defenses[31]. Similarly, SOD safeguards against ROS by transforming superoxide anions into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) [13]. Significantly, the Paraquat-exposed group showed reduced GSH levels, which lemongrass treatment effectively counteracted. In normal physiological conditions, enzymatic SOD and non-enzymatic GSH processes collaboratively restore redox imbalance due to oxidative stress[26].

The findings from this current study revealed the presence of flavonoids, phenols, saponins and tannins. There is a substantial increase in malondialdehyde levels, accompanied by a significant reduction in both GSH and the antioxidant enzyme SOD in the Paraquat-exposed group [6]. However, a remarkable reversal of these effects was clearly observed in the group treated with lemongrass. Importantly, the conclusions drawn from our study align harmoniously with the research results reported by Ikegwu[31].

4. CONCLUSION

While our study successfully replicated the induction of oxidative stress in RBCs through Paraquat exposure, numerous unexplored avenues await further investigation. Delving deeper into the intricate signalling pathways triggered by Paraquat-induced oxidative stress could significantly advance our understanding of cellular reactions and responses. In conclusion, our research not only provides valuable insights into the consequences of oxidative stress induction in red blood cells due to paraquat but also underscores the remarkable preventive impact demonstrated by Lemongrass (100µg). This effect could be attributed, at least in part, to the presence of bioactive phytochemicals like flavonoids, phenols, saponins and tannins, which collectively contribute to countering the oxidative damage provoked by paraquat.

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