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Original Article

Evaluation and estimation of diuretic activity of the linalyl acetate in the rats

Avaliação e estimativa da atividade diurética do acetato de linalila em ratos

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Abstract

This study aimed to explore the diuretic activity of linalyl acetate (LA). LA is an essential oil, it is an integral phyto-constituent of various plants. In this study, acute and chronic diuretic activities were explored by measuring the levels of different electrolytes and pH in the urine of experimental rats. Rats were divided into five groups. The control group was given 10 mg/kg normal saline, the treated group was given 10 mg/kg furosemide, and the remaining 3 groups received different doses of LA including 25, 50, and 75 mg/kg through intraperitoneal route, to determine its diuretic potential. Urine volume for acute diuretic activity was measured for 6 hours however for chronic diuretic activity was measured for 6 days. For a comparative study of LA with a control group and treated group with reference drug, diuretic index was used. Moreover, the underlying mechanism of the diuretic activity was also explored by comparing atropine, L-NAME, and indomethacin. The results of each group with 6 rats in each group were obtained by ± standard error of the mean of every group. Analysis of Variance (ANOVA) was used for statistical analysis. Results revealed that the LA 75 mg/kg dose showed comparable results as of furosemide. Moreover, this study revealed the involvement of muscarinic receptors to produce diuresis in comparison with atropine with very little involvement of prostanoids and no effect on NO pathway induced by indomethacin and L-NAME respectively. It is concluded that LA possess anti-diuretic potential. Muscarinic receptors might be involved in producing diuretic effects.

Keywords: urine electrolytes, furosemide, essential oils, L-NAME.

Resumo

Este estudo teve como objetivo explorar a atividade diurética do acetato de linalila (em inglês, LA). LA é um óleo essencial, um fitoconstituinte integral de várias plantas. Neste estudo, atividades diuréticas agudas e crônicas foram exploradas medindo os níveis de diferentes eletrólitos e pH na urina de ratos experimentais. Os ratos foram divididos em seis grupos. Diferentes doses de LA, incluindo 25, 50 e 75 mg/kg, foram usadas para determinar seu potencial diurético. O volume de urina para atividade diurética aguda foi medido por 6 horas, no entanto, para atividade diurética crônica foi medido por 6 dias. Além disso, o mecanismo subjacente da atividade diurética também foi explorado comparando atropina, L-NAME e indometacina. Os resultados de cada grupo com 6 ratos em cada grupo foram obtidos por ± erro padrão da média de cada grupo. A análise de variância (ANOVA) foi usada para análise estatística. Os resultados revelaram que a dose de 75 mg/kg de LA apresentou resultado comparável ao da furosemida. Além disso, este estudo revelou o envolvimento de receptores muscarínicos para produzir diurese em comparação com atropina com muito pouco envolvimento de prostanoides e sem efeito na via de NO induzida por indometacina e L-NAME, respectivamente. Conclui-se que os LA possuem potencial antidiurético. Os receptores muscarínicos podem estar envolvidos na produção de efeitos diuréticos.

Palavras-chave: eletrólitos de urina, furosemida, óleos essenciais, L-NAME.

1. Introduction

Diuretics are an important class of drugs used for the treatment of hypertension. Along with their therapeutic effects of balancing electrolytes and fluid concentration within the body, diuretics also have major side effects of hypokalemia, alkalosis, and disturbances between acid and base homeostasis. Therefore, it is challenging to formulate new agents for the treatment of hypertension with fewer side effects. Phytochemicals are those naturally occurring compounds that are used for the treatment of pathological conditions with low toxicity and adverse effects. Linalyl acetate (LA) refers to a phytochemical naturally occurring in the essential oil of many plants used for spice and flowers,

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including *Salvia sclarea* L., *Origanum majorana*, *Mentha citrata* Ehrh., *Myrtus communis* L. and *Citrus aurantium* L. (Elkousy et al., 2022). The chemical is usually one of the main components found within the essential oils of lavender. In regards to its structure, LA is a racemate that comprises equimolar amounts of both (S)- and (R)- Linalyl acetate. The chemical is additionally represented by the molecular structure $C_{12}H_{20}O_2$ with a molecular weight of 196.29 (Georgieva et al., 2022). Diuretics are used for the reduction of blood pressure and edema (Hsieh et al., 2018). Diuretics are used in combination with antihypertensive drugs including, angiotensin-converting enzyme inhibitors (ACEis) to facilitate their actions in patients with chronic kidney disease. Diuretics lower the complications of coronary vascular disease (Ter Maaten et al., 2020). Diuretics are used in hyperkalemia to maintain potassium levels in the blood (Hagengaard et al., 2020). patients' compliance is increased by long-acting diuretics along with antihypertensive drugs. Thiazide diuretics, loop diuretics and potassium-sparing diuretics are major three types of diuretics (Acelajado et al., 2019). Distal convoluted tubules, thick ascending limb of the loop of Henle, and collecting tubules are the site of action for thiazide diuretics, loop diuretics, and potassium-sparing diuretics respectively. All diuretics act on luminal space except spironolactone. Most diuretics are highly protein bound in nature so glomerular filtration has minimum effect on diuretic movement within the urinary compartment of the patients (Shah et al., 2017). LA has therapeutic properties in ischemic stroke by modifying intracellular Ca2+ concentration besides anti-oxidative properties (Hsieh et al., 2021). Despite the antihypertensive use and a preceding indication of efficacy and therapeutic prospective of LA, there are no studies that validate its use as a diuretic agent. So, this study was intended to evaluate the acute and chronic diuretic potential of LA as well as to find the diuretic mechanisms of the LA involved, in experimental rats. This study aims to evaluate the electrolyte excreting potential of LA. This study was performed in albino Wistar rats.

2. Materials and Methods

2.1. Materials

Furosemide, N-ω-Nitro-L-arginine methyl ester (L-NAME; NO synthase inhibitor), indomethacin, atropine, and linalyl acetate are purchased from Sigma–Aldrich Chemical Co. (St- Louis, MO, USA).

2.2. Animals

Both male and female albino Wistar rats of weight 200-250 g from the animal house of the University of Lahore were selected in this study. Rats were kept at controlled room temperature. Rats were placed in metabolic cages for a period of six days for acclimatization and adaptation to the environment before the investigational diuretic studies. The study design was developed for both acute and prolonged diuretic activity. Acute Diuretic activity was performed in 5 groups (A-E) of rats with six rats in each group. Group A was the control group that received normal saline 5 mL/kg whereas group B was taken as the treated group, and received furosemide 10 mg/kg. The remaining three groups (C-E) received different doses of 25, 50, and 75 mg/kg LA. The dose of LA was adjusted according to previous studies (Hsieh et al., 2018). Likewise, rats were divided into 5 groups with 6 rats in each group to study prolonged diuretic activity for a period of six days. This study was approved by the Institutional Research Ethics Committee (IREC), of Faculty of Pharmacy, University of Lahore, Lahore, Pakistan.

2.2.1. Evaluation of parameters related to diuretic effect

Acute diuretic action was determined by following Equation 1 (Rao et al., 2021):

$$\textit{Diuretic action} = \textit{urine volume of treated group} / \textit{urine volume of control group}$$

Diuretic activity was determined by following the Equation 2:

$$\textit{Diuretic activity} = \textit{diuretic action of treated group} / \textit{diuretic action of standard group}$$

2.3. Chronic diuretic activity

For chronic diuretic activity other five groups, comprising 6 animals in each group were taken for the chronic (6-day repeated-dose) diuretic activity. Rats were fasted 12 hours before treatment with free access to water. All animals received normal saline 5 mL/100g p.o of body weight to achieve water and salt uniformity. 60 minutes later group A was given vehicle (distilled water), group B received 20 mg/kg furosemide i.p, and groups C-E received 25, 50 and 75 mg/kg of LA respectively daily through i.p route for 6 days. Immediately after the administration of the drugs, rats were placed in metabolic cages. At days 2, 4, and 6 the urinary volume was recorded after every 6 hr. Urinary electrolytes excretion of Na^+, K^+, Cl^-,$HCO3^-$, pH were analyzed (Velmurugan et al., 2019).

2.4. Acute diuretic activity

The diuretic activity was calculated according to the method given by Lahlou et al. (2007). with slight modifications The rats were randomly divided into five groups, consisting of 6 animals in every group for the assessment of acute diuretic activity. Overnight fasted rats with free access to water and minimum distress were used for the evaluation of LA stated acute diuretic effects. Before treatment, rats were administered an oral dose of 5 mL/100g of normal saline solution (0.9%NaCl) to attain water and salt uniformity. After 60 minutes of administering normal saline, group A received 10 mL/kg p.o distilled water as a vehicle via oral gavage (Sayana et al., 2014). Group B received 100 mg/kg body weight of furosemide (Ref). Whereas the remaining 3 groups (C-E) were given 25, 50, and 75 mg/kg body weight of LA through the i.p route. These doses were selected based on previously published studies for LA (Kim et al., 2017). Instantly after this treatment animals were placed in individual metabolic cages. The urinary volume was recorded at the interval of 2 hours for a total period of 6 hours. The urine volume is expressed as mL/100g.

Electrolytes concentrations of sodium ion (Na^+), potassium ion (K^+), chloride ion (Cl^-), bicarbonates ($HCO3^-$), pH, urine excretion rate, and specific gravity were estimated from the collected urine sample of each rat of all the groups at the end of the experiment (6h) (Alam et al., 2012).

2.5. Evaluation of mechanisms involved in diuretic activity

To investigate the possible mechanisms of diuresis involved, groups received 60 mg/kg L-NAME, 5 mg/kg indomethacin, and 1 mg/kg atropine one hour before treatment and were maintained in standard conditions by following the procedure of acute diuretic activity (Zanovello et al., 2021).

2.5.1. Involvement of prostaglandins in the diuresis and saluresis induced by Linalyl acetate

After 60 minutes of oral administration of normal saline 5 mL/100g of body weight to achieve water and salt uniformity different groups of rats received 5 mg/kg indomethacin, a cyclooxygenase inhibitor through oral route, followed by 5 mL/kg oral administration of distilled water, 25, 50 and 75 mg/kg LA. The urinary volume (mL/100 mg) was measured 6h after treatment. Urinary concentrations of sodium, potassium, chloride, bicarbonates, pH, and specific gravity of urine samples were measured.

2.5.2. Involvement of muscarinic receptor in the diuresis and saluresis induced by Linalyl acetate

After giving 5 mL/100g normal saline orally for achieving water and salt uniformity, different groups of rats received 1 mg/kg atropine p.o, 1 h before experiments, followed by oral administration of 5 mL/kg distilled water and labeled as control group A, remaining groups (B-D) received LA with different doses of 25, 50 and 75 mg/kg i.p respectively through i.p route. The urinary volume (100 mg/mL) was measured 6h after treatment. The concentration of Na^+ and K^+, $HCO3^-$, pH and specific gravity of urine samples were also determined.

2.5.3. Involvement of nitric oxide in the diuresis and saluresis induced by Linalyl acetate

After giving 5 mL/100 g normal saline orally to achieve water and salt uniformity, different groups of rats received 60 mg/kg L-NAME orally 1 h before experiments, followed by oral administration of 5 mL/kg distilled water, 25, 50 and 75 mg/kg of both LA in three different doses. The urinary volume (mL/100 mg) was measured 6 h after treatment. Urinary electrolyte excretion of Na^+ and K^+, Cl^-, $HCO3^-$, pH, and specific gravity of urine samples were also determined (Zanovello et al., 2021).

2.6. Histopathology

After the dissection rats' kidney and pancreatic tissues were stored in a 10% formalin buffer solution. For histopathological examination slides were prepared by dehydration with ethanol and embedded in paraffin. hematoxylin and eosin staining was applied on 5µm sections of pancreatic and kidney tissues, using a rotary microtome. These stained sections were studied under a microscope (Ahmed et al., 2021).

2.7. Statistical analysis

The results were presented as mean ± standard error of the mean (S.E.M) of 6 rats in each group. The statistical analysis was done by using the GraphPad Prism version 7.00 for Mac (GraphPad Software, La Jolla, CA, USA), by using the one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test and two-way ANOVA followed by Tukey post hoc test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of linalyl acetate on urinary volume of rats

Urine volume was increased in groups treated with LA at doses of 25, 50 and 75 mg/kg. Group B was taken as the control drug for comparison with the diuretic effect of LA. Diuretic effects in group D during 1st hour with LA at a dose of 50 mg was comparable to the standard treated group B. Average urine volume was greater in LA after administration at the same concentration as shown in Table 1.

3.2. Effect of linalyl acetate on urinary parameters of rats

Urinary parameters such as urine electrolytes, including Na^+, K^+, Cl^-, $HCO3^-$, Urine pH, and specific gravity were also analyzed to evaluate urine osmolality. Urine osmolality level was also increased in standard control group B as well as in LA treated groups C-E groups as compared to normal control Group A. Whereas no significant changes were observed in pH and specific gravity of the urine as shown in the figure. Moreover, LA treated group D at a dose of 50 mg/kg was able to increase the level of Na^+. Similarly, the level of $Cl^-$and $HCO_3^-$was also increased by LA at a dose

Table 1. Showing the urine volume in the treated and non-treated rats.

Groups	Urine volume (Stojanović et al., 2019)	Diuretic action	Diuretic activity
Normal	1.50 ± 0.10	1	0.39
Standard	3.80 ± 0.20 ***	2.53	1
LA25 mg	2.870± 0.10**	1.91	0.75
LA50 mg	3.10± 0.21***	2.06	2.06
LA75 mg	3.50 ± 0.15***	2.33	2.33

One-way Anova followed by Dunnett's post hoc test was done to find out the level of significance. **p<0.01; ***p<0.001.

of 50 mg/kg in group D. Similar effect for LA was observed in K^+ excretion. Whereas at a dose of 25 mg/kg in group C, LA was unable to eliminate urine electrolytes including K^+, Na^+, and Cl^- ions. The same result was observed in group B with the standard treated group as shown in Figure 1.

3.3. Effect of linalyl acetate on urine volume and electrolyte excretion

Chronic administration of 25, 50 and 75 mg/kg LA for 6 days markedly enhanced diuresis starting from the first day of treatment (Figure 1). There was also a significant increase in Na^+, K^+, and Cl^- ions excretion on the 6th day after administration of both LA at 25, 50 and 75 mg/kg. These results were very comparable to diuretic furosemides.

3.4. Diuretic effect of LA after pretreatment with atropine, indomethacin and L-NAME

Diuretic effects including the elimination rate of different electrolytes, caused by different doses of LA completely inhibited by the pretreatment of atropine as shown in Figure 2. Whereas indomethacin has no effect on the diuresis induced by the higher doses of LA in group E as shown in Figure 3. L-NAME did not change the diuresis produced by LA at a dose of 50, 75 mg/kg in group D and E as shown in Figure 4. These had produced the same diuretic effect on L-NAME pretreated rats. Whereas, rats treated with atropine prior to treatment with 50 mg/kg and 75 mg/kg LA have synergistic blocking effect on Na^+ and K^+ ions excretion in urine.

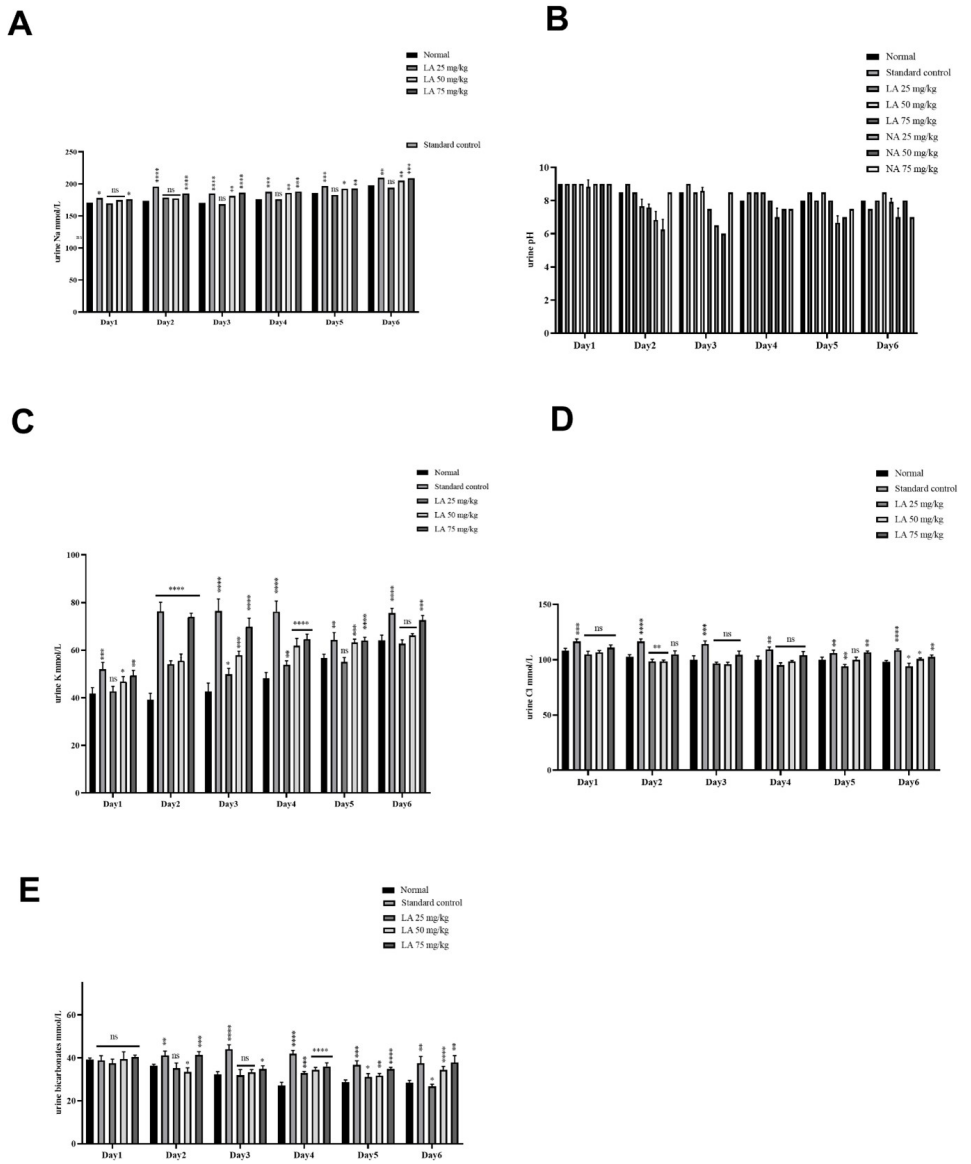

Figure 1. Showing the levels of the urinary ions (A) Na, (B) pH, (C) K, (D) Cl, (E) HCO_3. Values are presented as mean ± SEM. (n= 6) for each group. Data were analyzed by two way Anova applying Dunnet's multiple comparison test shows significant differences when compared to group A. Whereas, *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001; ns, non significant.

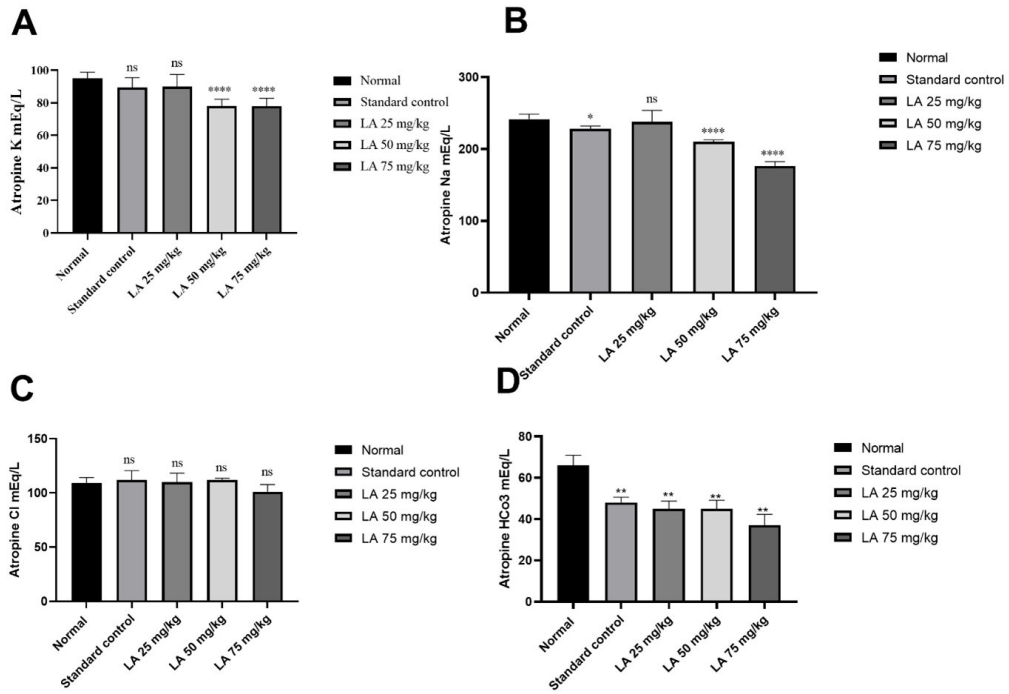

Figure 2. Showing the levels of the urinary ions in the atropine pretreated rats (A) Na, (B) K, (C) Cl, (D) HCO_3. Values are presented as mean ± SEM. (n= 6) for each group. Data were analyzed by Two way Anova applying Dunnet's multiple comparison test shows significant differences when compared to group A. Whereas, *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001; ns, non significant.

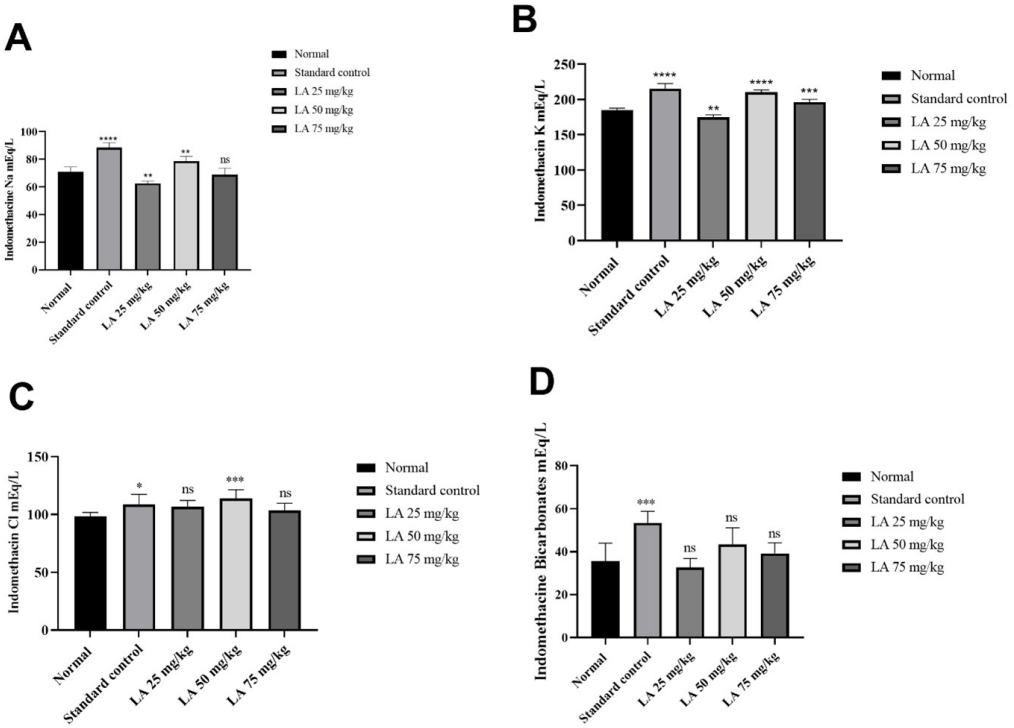

Figure 3. Showing the levels of the urinary ions in the indomethacin pretreated rats (A) Na, (B) K, (C) Cl, (D) HCO_3. Values are presented as mean ± SEM. (n= 6) for each group. Data were analyzed by Two way Anova applying Dunnet's multiple comparison test shows significant differences when compared to group A. Whereas, *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001; ns, non significant.

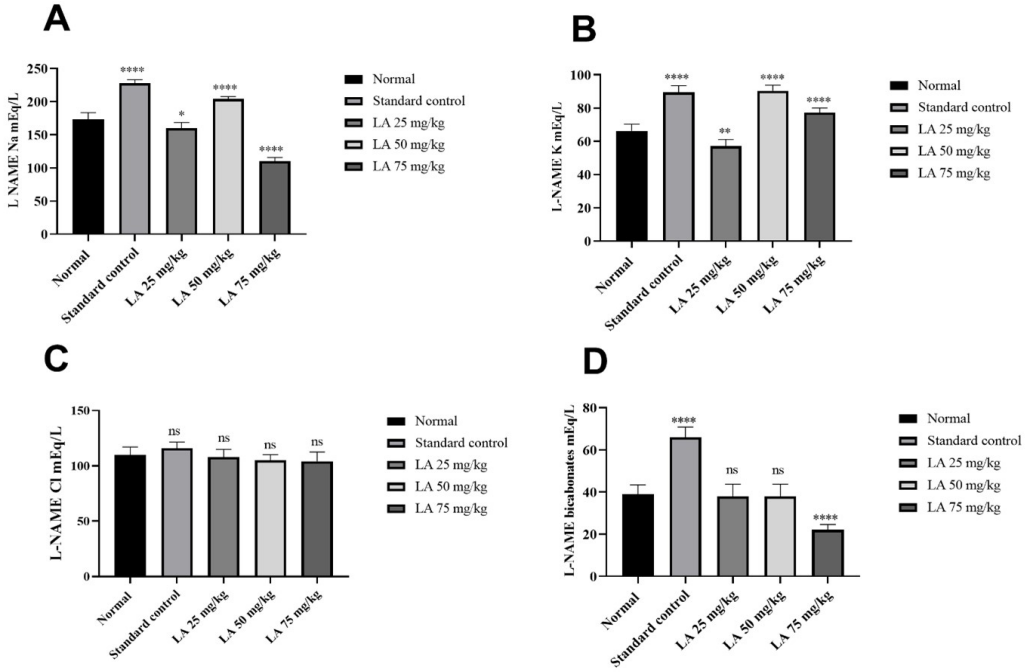

Figure 4. Showing the levels of the urinary ions in the L-NAME pretreated rats (A) Na, (B) K, (C) Cl, (D) HCO_3. Values are presented as mean ± SEM. (n= 6) for each group. Data were analyzed by two-way ANOVA applying Dunnet's multiple comparison test shows significant differences when compared to group A. Whereas, *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001; ns, non significant.

3.5. Histopathology

Normal rats show healthy renal tissues in histopathology of glomerulus. A visible lumen of renal tubules was seen along with clear bowmen's capsule and glomerulus structure with nuclei within the cell were also shown distinctively. Diabetic rats show significant occurring changes in the histopathology of the kidney glomerular and tubules. Narrowing of the Bowmen's capsule and necrosis of the glomerulus was seen in DN rats.

Tissue repair and improvements were shown within the treated rats' histopathological studies. Moreover, substantial improvements were shown in treatment with 75 mg/kg LA at the highest dose and with glimepiride. Narrowing of Bowman's space and glomerulus occur with progressive improvements of the tubular epithelium. sizes were narrowed down, and the tubule epithelium also displayed enhancement. Notably, 50 and 75 mg/kg LA exhibit a more substantial effect as compared to a lower dose of 25 mg/kg LA as shown in Figure 5.

4. Discussion

Worldwide more than 60% of the population is affected by hypertension and its comorbidities of nephropathy, retinopathy, and liver diseases. Diuretics are drugs that can maintain body fluid amount by increasing urine and salt excretion. Hence these medications can be used to cure several clinical abnormalities, such as hypertension, and cardiovascular and nephropathy complications (Arumugham and Shahin, 2021). Loop diuretics, such as furosemide, can improve urine flow while also functioning as a potent diuretic, boosting salt and chloride output in the urine. Actions of antihypertensive drug is facilitated by diuretics (Agarwal et al., 2021). Hyperkalemia can also be treated with the use of diuretics. patient's compliance with drug therapy is enhanced by long-acting diuretics. Unfortunately, existing diuretics exhibit many adverse effects that need to be minimized by introducing new therapeutic agents to control blood pressure.

This study intends to systematically assess the diuretic effects of LA in rats. Previous studies have less evidence about the effectiveness and safety of diuretics in many clinical situations. So, To guide clinicians about the right time to switch diuretics in patients, there should be further research on this important class of drugs. Furthermore, previous studies provide partial information about appropriate titrating doses in hypertensive patients (James et al., 2014). Therefore, in this study, we have evaluated the possible diuretic potential and mechanism of LA. Commonly used loop diuretics are; Bumetanide, furosemide, and torsemide. The common use of loop diuretics is to decrease fluid overload due to heart failure and advanced kidney failure. loop diuretics in combination with thiazide diuretics also minimize drug-induced edema by minoxidil and other vasodilators (Sinha and Agarwal, 2019).

As furosemide is a loop diuretic, it was taken as a standard control group. Hyperkalemia is treated by the use of furosemide decreasing the level of potassium in serum after its excretion through urine by acting on the loop of Henle. Furosemide was taken as a reference drug.

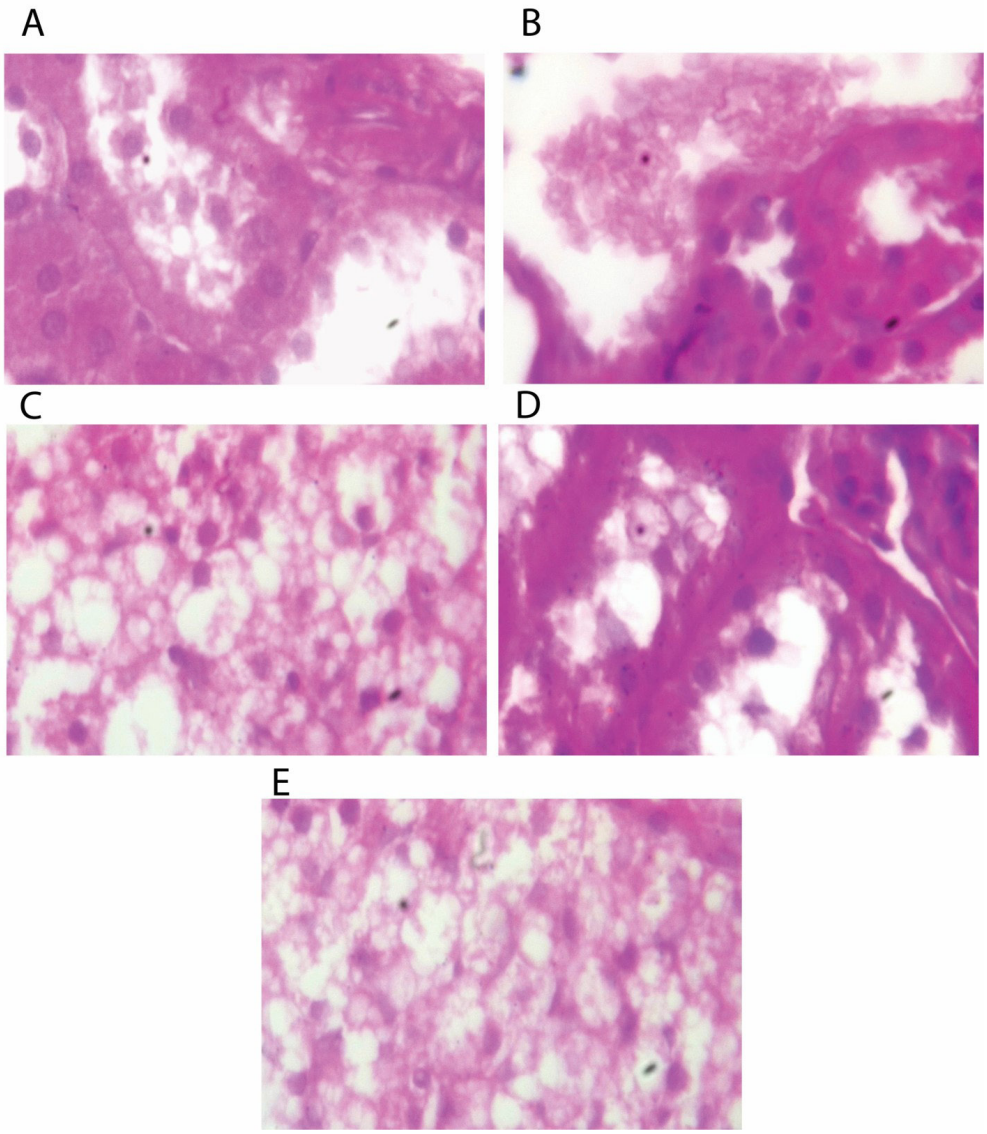

Figure 5. Showing the histopathological images (40X) (A) normal control (B) standard control (C) LA 25 mg/kg (D) 50 mg/kg (E) 75 mg/kg.

It acts on the thick ascending limb of the loop of Henley and inhibits $Na^+/K^+/2Cl^-$ symporter to enhance urine output and excretion (Yang et al., 2021). This study shows that LA enhanced the urine volume along with the excretion of electrolytes in a dose-dependent manner. In lower doses, 25 mg/kg of LA possesses no significant natriuretic or saluretic effect. Higher doses, LA 50 and 75 mg/kg produced significant diuresis and saluresis including the significant excretion of Na^+, K^+, Cl^- and HCO_3 in the urine, which represents dose-dependent diuretic effect of LA. This could be analyzed that the diuretic effect produced by 50 mg/kg LA might be due to the involvement of [HCO3+/H+] and the [Na+/H+] antiporter, a similar effect observed in comparison with high ceiling diuretics. Nevertheless, LA at a dose of 50 mg/kg and 75 mg/kg were able to produce natriuresis as well as saluresis. Moreover, LA at 50 and 75 mg/kg was able to increase the excretion of K^+ and Na^+ in urine. Furthermore, neither LA nor furosemide were able to excrete Cl^- in urine. Moreover, LA at 50 and 75 mg/kg was able to increase the excretion of K^+ and Na^+ in urine. An important factor involved in hypertension is sodium ions. A high level of sodium within the blood leads to an increase in blood pressure, which could be minimized by the use of LA by its potential to excrete sodium ions. Hence, it proves LA is a blood pressure-lowering agent with diuretic potential. Kaliuretic effect was observed at 50 mg/kg dose of LA which represents its loop diuretic-like potential. Increased excretion ratio of sodium ions as compared to potassium ions indicates LA a good quality diuretic with safe use in hyperkalemic patients (Chen et al., 2019). Our findings suggest that LA can be used in renal-compromised patients to reduce complications by aiding in decreasing fluid volume and electrolyte concentrations in the blood due to its diuretic

potential. A further experimental study was performed to determine the most probable diuretic mechanism of LA. Pretreatment with L-NAME, indomethacin, and atropine was done in each rat. Nitric oxide (NO) is generated from its enzyme nitric oxide synthetase. This NO regulates renal functions of glomerular filtration along with vascular tone of the renal artery. Moreover, NO and prostaglandins (PG) are also involved in maintaining blood pressure by improving vascular tone. Any disturbance of NO production and release disturbs blood pressure regulation by abnormality of balancing in salt and water excretion induced by NO (Younis et al., 2020). L-NAME which is NO synthesis inhibitor was not able to alter the diuresis produced by LA at higher doses. Atropine and indomethacin being antimuscarinics and prostaglandin inhibitors were used to evaluate the diuretic potential of LA. atropine pretreatment groups were unable to induce diuresis and saluresis, which highlights the muscarinic receptors' involvement in the LA diuretic mechanism due to the endothelium vasodilation which raises capillary blood flow and ultimate increase in diuresis by washing out of hypertonic medullary interstitial (Younis et al., 2021). Indomethacin has very little significant effect only on the diuresis produced by 75 mg/kg LA. These findings indicate that there is little involvement of prostanoids which could be overcome by higher doses of LA at 75 mg/kg. This study represents the significance of LA in lowering blood pressure with a major involvement of muscarinic receptors. More study is required to investigate the efficacy of LA in detail during different pathological conditions of the renal and cardiovascular systems. Also, toxicological studies on a more prolonged model need to be carried out to evaluate possible toxicities of LA.

5. Conclusion

Our findings suggested that LA can be used in renal compromised patients to reduce complications by aiding in decreasing fluid volume and electrolytes concentrations in blood. Predominant diuretic effect might be due to the involvement of muscarinic receptors. The muscarinic receptors cause endothelial vasodilation hence produces the diuretic effect. Further studies are suggested to explore the exact underlying diuretic mechanism.

Abbreviations

Linalyl acetate (LA), Furosemide, N-ω-Nitro-L-arginine methyl ester (L-NAME).

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