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ANALGESIC AND ANTI-LITHIASIC EFFECTS OF MOROCCAN *Citrus aurantium* FLOWERS AND FRUIT AQUEOUS EXTRACTS

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ABSTRACT

Citrus aurantium holds widespread use in Moroccan society as a remedy for various diseases, owing to its significant pharmacological properties. The objective of this study was to investigate the antioxidant activity, analgesic potential, and anti-lithiasis properties of the aqueous extracts of both the fruits and juice of *Citrus aurantium*. To achieve this, we employed various techniques: antioxidant activity was assessed using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and reducing power assays (FRAP), while the antinociceptive effect of the plant was evaluated through writhing and hotplate tests on mice. The urolithiasis model was induced in rats through the administration of ethylene glycol and ammonium chloride, and assessments were made based on variations in body weight, kidney histopathology, and biochemical analysis using urea and creatinine. Our findings demonstrated significant antioxidant activity in both extracts (flowers and juice) of *Citrus aurantium*, represented by DPPH values of 5.42±0.20 and 2.87±0.42, and FRAP values of 4.23±0.05 and 1.67±0.13 in flowers and juice, respectively. The obtained results also showed that *Citrus aurantium* extracts significantly reduced the number of writhes and increased the latency time in response to a thermal stimulus compared to the control group ($p \leq 0.001$). Both extracts (flowers and juice) of *Citrus aurantium* exhibited a protective effect on the kidneys by preventing the formation of oxalo-calcium crystals. The histopathological study of the kidneys in the groups treated with *Citrus aurantium* revealed a marked reduction in abnormalities observed in rats treated with Ethylene Glycol. Additionally, urea and creatinine values were reduced compared to the control group ($p \leq 0.001$), signifying an important anti-urolithiasic activity. Overall, *Citrus aurantium* demonstrated potent antioxidant, analgesic and antilithic activities, suggesting its potential as a valuable natural source of bioactive compounds with various therapeutic applications.

Keywords: *Citrus aurantium*; Uro-lithiasis; Histopathological examination; Biochemical parameters; Analgesic effect.

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INTRODUCTION

The utilization of herbal remedies for treatment can be traced back to ancient times (Newman and Cragg., 2020) . Recently, there has been a resurgence of interest in medicinal plants, driven by the compelling results achieved by scientists in the field of phytotherapy (Baslam *et al*., 2023). Medicinal plants find widespread use globally in combating various diseases and their associated symptoms (Howes *et al*., 2022). Renal lithiasis, a multifactorial condition, arises from an alteration in the normal crystallization conditions of urine within the urinary tract (Marhoume *et al*., 2021). In a healthy individual, the residence time of urine in the urinary tract does not lead to the formation of crystals, or if formed, they are typically so minuscule that their elimination occurs without causing any symptoms (asymptomatic crystalluria) (Khan *et al*., 2018). When the normal conditions for urine crystallization undergo alterations, the rate of crystal nucleation and growth may escalate to a point where the crystals become challenging to eliminate owing to their size (Grases *et al*., 2006). Over the last three decades, there has been a noticeable rise in the incidence of kidney stones among both adult men and women. Pain, a ubiquitous symptom across various illnesses, is particularly pronounced in cases of acute urinary lithiasis, where flank pain is intricately linked to the aforementioned pathological processes, presenting a management(Marhoume *et al*., 2021).

Medicinal plants offer a potential alternative to anti-lithiasic drugs owing to the presence of various active compounds and minimal side effects (Bencheikh *et*

al., 2021). It has been postulated that plants possessing anti-lithiasic characteristics exert their effects through antioxidant capacities, which mitigate the toxicity generated by free radicals implicated in the initiation and progression of urolithiasis (Youn *et al*., 2017). Numerous prior studies have established a discernible correlation between the anti-lithiasic effect and the presence of phenolic compounds, known for their potent antioxidant properties (Khan *et al*., 2018; Maksoud *et al*., 2021; Marhoume *et al*., 2021).

Citrus aurantium, a plant belonging to the Rutaceae family, exhibits distinctive properties that afford it versatile applications. Phytochemical analysis of *Citrus aurantium* has uncovered the presence of vitamins, minerals, phenolics, terpenoids, and flavonoids (Khan *et al*., 2018; Maksoud *et al*., 2021). The abundance of biologically active constituents in *Citrus aurantium* imparts significant physiological and pharmacological attributes to the plant, including analgesic and anti-inflammatory activities (Maksoud *et al*., 2021). Moreover, the plant is renowned for its robust antioxidant activity, attributed to its inherent capacity to scavenge free radicals and disrupt radical chains (Marhoume *et al*., 2021).

Building upon the remarkable properties of *Citrus aurantium*, the current study aims to extend our understanding by evaluating the effectiveness of aqueous extracts (flowers and juice) of *Citrus aurantium*. Notably, despite the plant's known bioactive potential, the antilithiatic effect remains an aspect that has yet to be comprehensively investigated. To our knowledge, there is a notable gap in the existing literature regarding the evaluation of the antilithiatic properties of *Citrus aurantium*. Therefore, this study seeks to bridge this gap by assessing the potential preventive impact on stone formation and the concurrent efficacy in pain treatment.

MATERIALS AND METHODS

Plant material: The flowers and fruits of *Citrus aurantium* were harvested during flowering period, from the wild in its natural habitat at Aghmat, Marrakech region, Morocco (31° 25’21’’latitude N/ 7° 48’4’’ Longitude W). The plants were initially authenticated by botanist Professor A. Ouhammou and pharmacologist Professor A. Chait from the Faculty of Sciences at Semlalia, Cadi Ayyad University. The plants were then stored under voucher specimen MARK-AC010 in the herbarium of the Department of Biology, Faculty of Sciences Semlalia, Cadi Ayyad University, Marrakech, Morocco.

Then, fruits were immediately pressed with a hand-held citrus press followed by filtration process. Juice extract was lyophilized and flowers were dried then crushed. Obtained powder was mixed with distilled water.

Animal material: Male Swiss mice (n=24) weighing 25-30g and Male Sprague Dawley rats (n=24) weighting 180 and 260 g were used to evaluate pharmacological assays. The animals were housed in the animal facility of Faculty of Sciences Semlalia and kept at constant room temperature (25 ± 1 °C) with a 12 hours light/dark cycle and had free access to water and food. Animals were acclimatized in the laboratory conditions one hour before all experiments.

Pharmacological assays

Antioxidant activity

Determination of DPPH radical scavenging activity: In accordance with the method employed by Aitbaba *et al*. (2023), The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay was used to determine the antioxidant power of the aqueous extracts. Briefly, 2 mL of methanol solution of DDPH (60M) was mixed with 10 µL of each sample at various doses. Thirty minutes later of incubation, the absorbance at 570nm was measured. Quercetin and BHT were utilized as positive control.

Determination of ferric reducing ability power (FRAP) activity: The FRAP test was carried out in accordance with a previously established method (Kabdy *et al*., 2024) . The assay involves the prevention of the formation of Fe(II)-ferrazine complexes when samples interact with ferrous iron. In the procedure, a mixture of distilled water (1 mL), phosphate buffer (2.5 mL; 0.2 M; pH = 6.6), and potassium ferricyanide ($K_3[Fe(CN)_6]$) (2.5 mL; 1 %) was combined with 0.5 mL of solutions of varying concentrations. After incubating for 30 min, the mixture was supplemented with 2.5 mL of distilled water, 2.5 mL of 10 % trichloroacetic acid, and 0.5 mL of $FeCl_3$. The absorbance was read at 700 nm. Quercetin and BHT were used as positive controls.

Anti-lithiasis activity test: In this test, renal lithiasis model was created by providing 0.75% ethylene glycol (EG) in the drinking water (to induce the formation of calcium oxalate crystals in the kidneys) with the 2% ammonium chloride (NH_4Cl) (Marhoume *et al*., 2021). Crystals were developed 7th day later. The administration of the extracts to rats was carried out orally and body weight of rats was measured daily during period of treatment and divided into four groups of six animals.

After 3 days of acclimation, the 24 male Sprague Dawley rats were randomized in 04 groups of 6 individuals as follows:

-Control group : the untreated control group (or vehicle control) received only distilled water during the ten days.

-Control EG group : the lithiasic control group received distilled water supplemented with 0.75%

ethylene glycol (EG) and 2% ammonium chloride (AC), during the ten days

- *Citrus aurantium* flowers extract group: received

distilled water supplemented with 0.75% ethylene glycol (EG) and 2% ammonium chloride (AC) and concomitantly, 1 mL/day of 200 mg/kg of *Citrus aurantium* flowers aqueous extract, dissolved in distilled water, respectively, during the ten days.

- *Citrus aurantium* juice extract group: received distilled water supplemented with 0.75% ethylene glycol (EG) and 2% ammonium chloride (AC) and concomitantly, 1 mL/day of 200 mg/kg of *Citrus aurantium* juice aqueous extract, dissolved in distilled water, respectively, during the ten days (Table 01).

Table 1: Schematic time line of anti-lithiasis activity test.

	Drink liquid	Treated groups
Control	Water	0,9% NaCl
Control EG	EG (0,75%) + NH_4Cl (2%)	0,9% NaCl
***Citrus aurantium* flowers extract**	EG (0,75%) + NH_4Cl (2%)	Extract 200 mg/kg
***Citrus aurantium* juice extract**	EG (0,75%) + NH_4Cl (2%)	Extract 200 mg/kg

Biochemical analysis: Biochemical parameters (urea and creatinine) were estimated to verify possible variations. Initially, the animals were anesthetized by intraperitoneal injection of 0.6% chloral hydrate (10 mL/100 g) of body weight, and blood samples were then obtained using capillary hematocrit tubes through the jugular vein of the animals. The collected blood was placed in dry tubes (2 mL) and centrifuged at 3000 rpm for 10 minutes. Subsequently, serum was utilized to measure urea and creatinine levels. The assays were conducted following the protocols provided with the commercial CHRONOLAB kits applied to the BA-88A Semi-Auto Chemistry Analyzer (Mindray-China).

Histological study: All groups were used for histological examination. The rats were sacrificed by cervical dislocation at last day of treatment. The kidneys were dissected and fixed in 10% formalin solution overnight. Then organs were subjected to dehydration in a series of graduated alcohols and incorporated into paraffin. Sections 4 to 10 µm thick were stained with Hematoxylin-Eosin (HE) (Baslam *et al*., 2024).

For the histopathological observations, a comprehensive examination was performed on each section. The histopathological profiles of different experimental groups, including changes in glomeruli, tubules, inflammatory infiltration, and hemorrhage, were meticulously compiled and averaged from observations made on six rats per group. The severity of alterations was categorized as follows: (-) for no alteration, (+) for mild alteration, (++) for moderate alteration, and (+++) for marked alteration.

Analgesic activity: The analgesic activity of *Citrus aurantium* extracts, derived from both flowers and juice, was evaluated through writhing and hot plate tests on four groups of mice, each group comprising six animals as follows:

Group 1: mice treated with saline solution (10 mL/kg; orally).

Group 2: mice treated with reference drugs: Aspirin (200 mg/kg) and Morphine (10 mg/kg) for writhing and hotplate test respectively.

Group 3: mice treated with aqueous extract of *Citrus aurantium* flowers (200 mg/Kg, orally).

Group 4: mice treated with aqueous extract of *Citrus aurantium* juice (200 mg/Kg, orally).

Acetic acid induced abdominal constriction: The writhing test was employed to assess the nociceptive peripheral response to a chemical stimulus, following the method described by Aitbaba *et al*. (2023). In brief, acetic acid (0.6%) was intraperitoneally administered to mice (0.1 mL/10 g). Thirty minutes prior, all groups (n=6 each) were treated with a saline solution, aqueous extracts of *Citrus aurantium* (200 mg/kg), and Aspirin as the reference drug (200 mg/kg). Subsequently, the mice were placed in appropriate cages for the observation and counting of abdominal constrictions over a period of 30 minutes

Hotplate test: The hotplate test was used to evaluate the central nociceptive response in mice following thermal stimuli. Mice were placed in the center of the hot metal plate (50-55 ° C ± 0.1 ° C). This test was carried out immediately 30 min after administration of aqueous extracts of *Citrus aurantium*. Reaction time was measured by latency, paw licking or jumping (Ripoll, 2005). Twenty seconds were considered as cut off time.

Statistical analysis: Data were presented as mean ± SEM. Differences between control and treatments were analyzed using ANOVA followed by Kruskal Wallis or Tukey's tests followed by post hoc-hoc analysis.

RESULTS

Antioxidant activity: The in vitro antioxidant activity of aqueous extracts was assessed using various tests, including DPPH and FRAP assays. The results obtained demonstrate that *Citrus aurantium* possessed a noteworthy antioxidant capacity. As shown in Table 1, the IC50 values for DPPH and FRAP were 5.42 and 4.23 mg/mL, respectively, for the flower extract. On the other

hand, the antioxidant capacity of the juice extract was higher in comparison with the former.

Table 1: Antioxidant capacity of aqueous extracts of *Citrus aurantium.*

	Citrus aurantium		Standard antioxidant	
	Flowers extract	**Juice extract**	**Quercitine**	**BHT**
DPPH	5,42±0,20	2,87±0,42	0,10±0,06	0,25±0,09
FRAP	4,23±0,05	1,67±0,13	0,05±0,01	0,02±0,01

Urinary lithiasis

Body weight: The results depicted in Figure 1 show that the body weight of rats treated with EG (positive control) decreased over the 10-day treatment period compared to the negative control group. The group treated with the juice extract exhibited a decline in weight during the first two days but started to increase in the subsequent days. Conversely, the group treated with the flower extract displayed a significant variation in body weight.

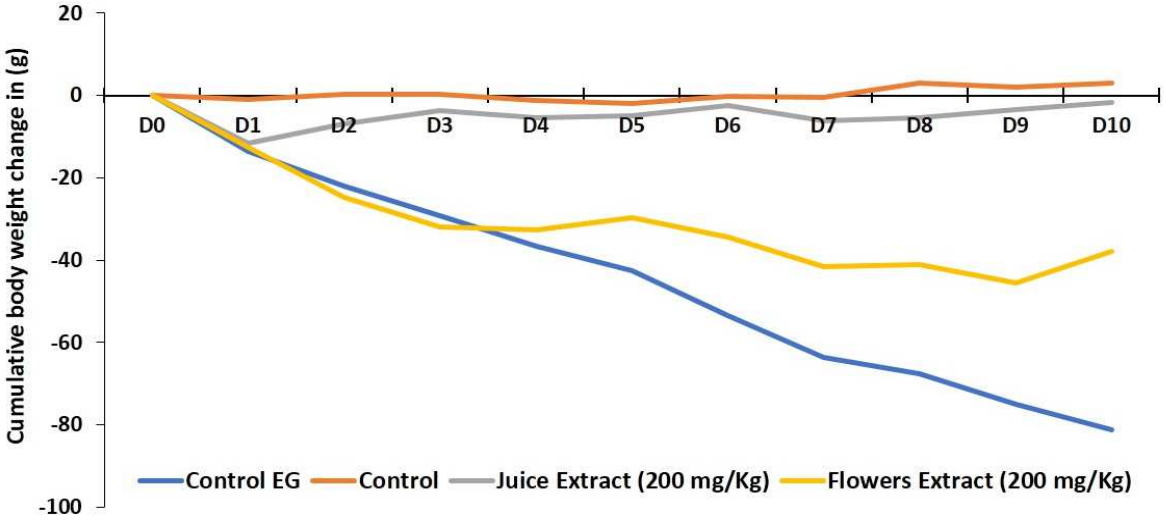

Figure 1: Variation of body weight of the experimental animal groups for 10 days post treatment.

Biochemical parameters: The statistical analysis revealed a highly significant decrease ($p \leq 0.001$) in the concentration of Urea (Figure 2a) and Creatinine (Figure 2b) in the groups treated with both extracts of *Citrus aurantium* compared to the group treated with ethylene glycol. This decrease in these biological indicators indicated the restoration of renal function.

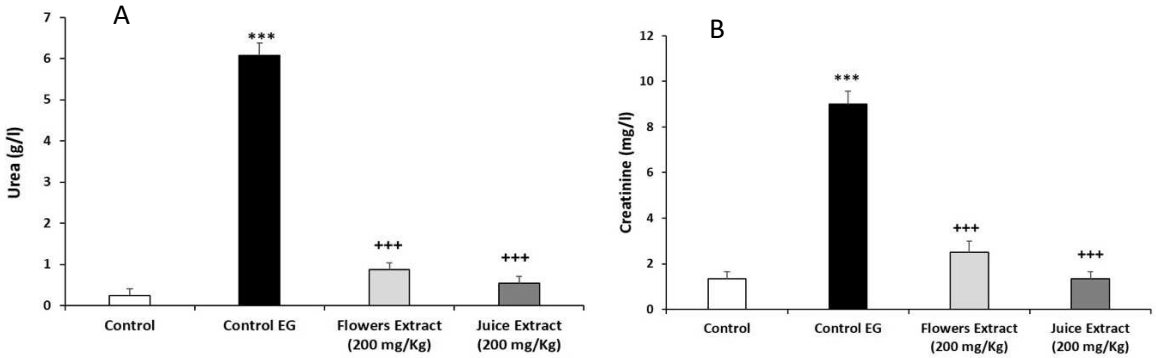

Figure 2: (a)The Urea level in g/l and (b) The Creatinine level in different groups of rats. The results are expressed as mean ± SEM. ***$p \leq 0.001$compared to control group. +++$p \leq 0.001$ vs Control EG.

Histopathology assessment: The histopathological study of the kidneys in the EG-treated group revealed morphological disorganization of the glomeruli, tubules, mononuclear inflammatory infiltration, and hemorrhage

(Figure 3). In contrast, the histopathological study of the kidneys in the groups treated with both extracts of *Citrus aurantium* showed a marked reduction in the abnormalities observed in the rats treated with EG. This finding confirms the preventive potential of the evaluated extracts.

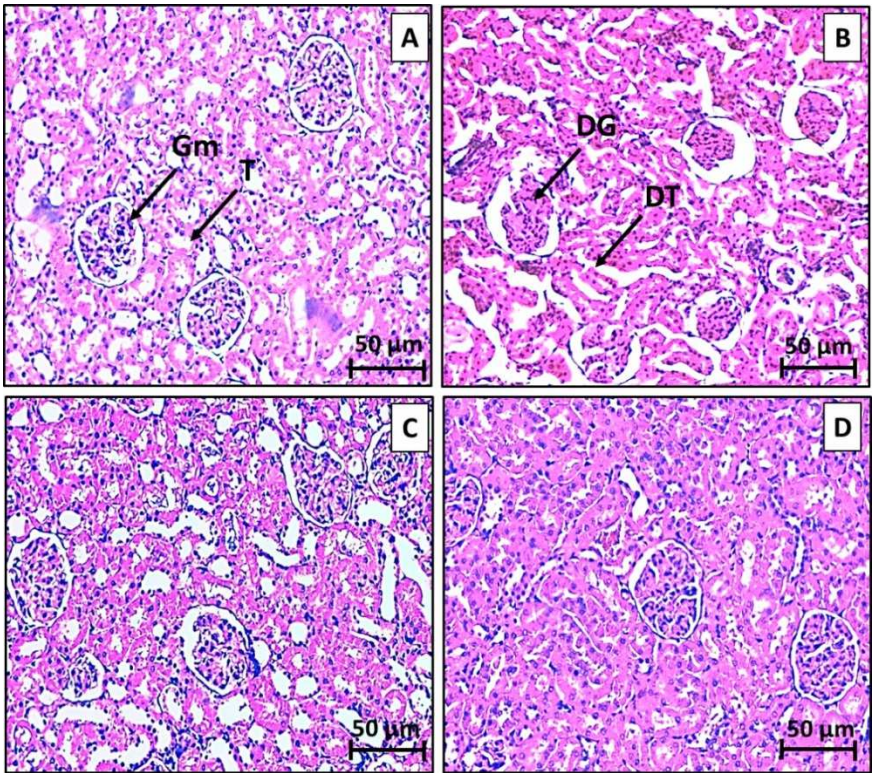

Figure 3: Microscopic aspect of kidneys of different groups (Figure A: Normal untreated rat; Figure B: group receiving EG; Figure C: group treated with *Citrus aurantium* flower extract (200 mg/kg); Figure D: group treated with *Citrus aurantium* fruit's extracts (200 mg/kg). (Gm: Glomerulus, DG: glomerular disorganization, DT: tibular disorganization,T: tubule).

Table 2: Histopathological profiles of different experimental groups. Changes were compiled and averaged from 6 rats per group. (-): no alteration; (+): mild alteration; (++): moderate alteration; (+++): marked alteration.

	Control	Control EG	Flowers extract (200 mg/kg)	Juice extract (200 mg/kg)
Morphological disorganization of the tubules	-	+++	+	-
Morphological disorganization of the glomeruli	-	+++	+	+
Mononuclear inflammatory infiltration	-	+++	+	-
Hemorrhage	-	+++	+	-

Nociception activity

Writhing test: In this test, the extracts tested (200 mg/kg) decreased significantly ($p \leq 0.01$) the number of writhes induced by acetic acid. The juice extract showed the highest activity against abdominal spasms. This effect is comparable to that obtained by the reference drug (Figure 4).

Hotplate test: The results obtained from Figure 6 indicated a significant effect of the juice extract on nociceptive sensitivity induced by thermal stimulus. Oral administration of the juice extracts increased the reaction time at a dose of 200 mg/kg. A similar effect was observed for the flower extract, although to a lesser extent compared to the juice extract.

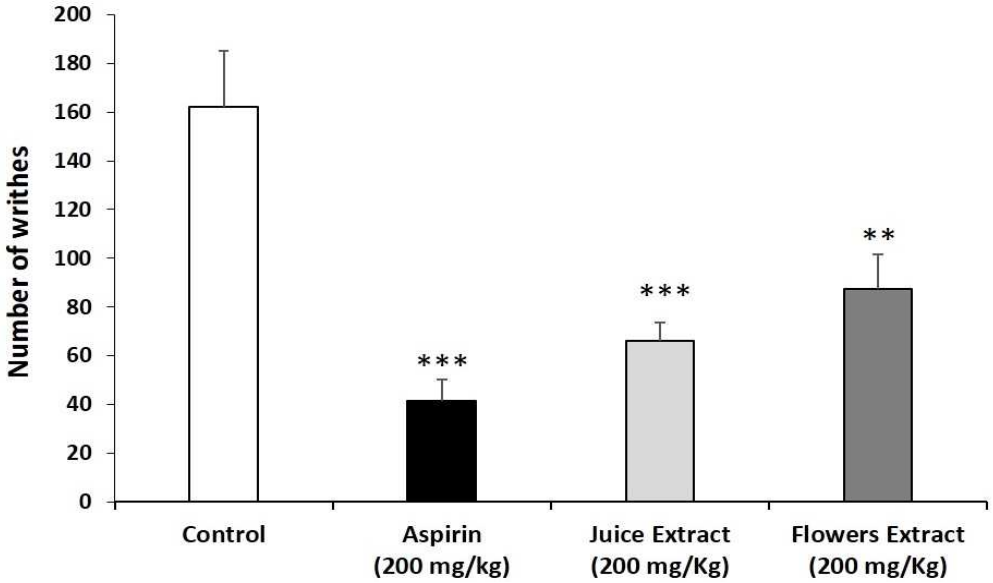

Figure 4: Effect of *Citrus aurantium* extracts on the number of writhes caused by acetic acid injection. Results are presented as mean ± SEM ** p<0.01 vs control.

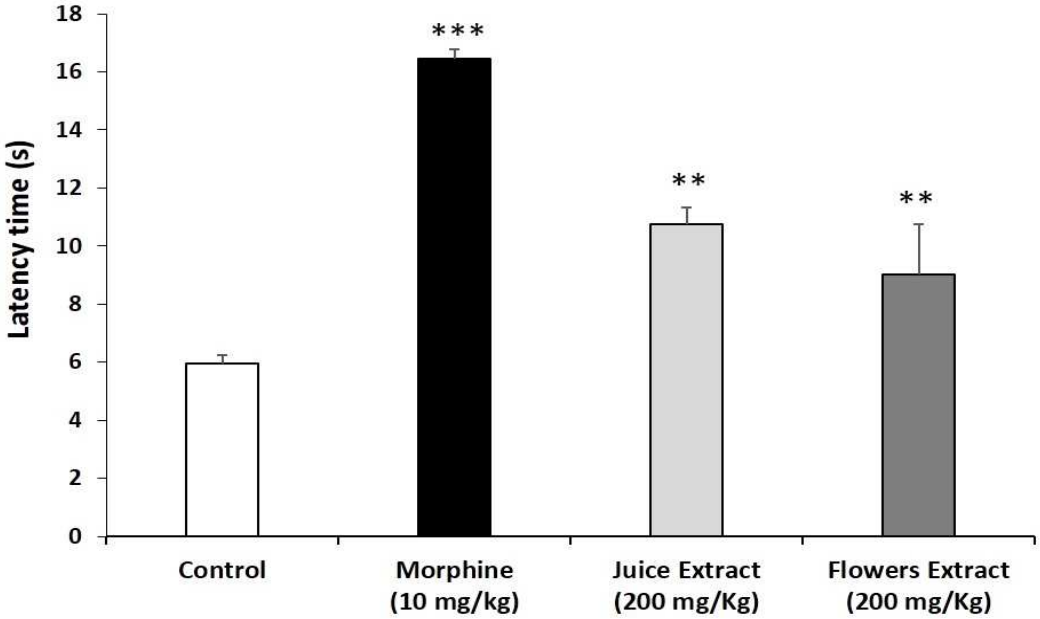

Figure 5: Effect of *Citrus aurantium* extracts on latency time in the hotplate test. The results are presented as mean ± SEM *** p<0.001 vs control.

DISCUSSION

Even before recorded history, people employed plants for therapeutic purposes, and this practice is still widely embraced today (Parasuraman *et al*., 2014). Several medicinal plants, including *Citrus aurantium*, have therapeutic virtues whose knowledge is currently limited. Therefore, our aim was to explore the antioxidant, analgesic, and anti-urolithiasis activities by comparing extracts from both the flowers and juice of *Citrus aurantium*. In our investigation, we first explored the antioxidant activity of *Citrus aurantium* flowers and juice extracts. According to our results, the *Citrus aurantium* fruit extract exhibited a higher potential to reduce the rate of oxidation (IC50=2.87 mg/mL) compared to the *Citrus aurantium* flowers extract (IC50=5.42 mg/ml) using the DPPH assay. Similarly, in the reducing power-scavenging assay, the fruit extract (IC50=1.67 mg/mL) showed a more potent effect compared to the flowers extract (IC50=4.23 mg/mL).

The antioxidant property was attributed to the

chemical composition of the juice which is rich in phenolic acids (86%) and their derivates (Tounsi *et al*., 2011). Indeed, the total phenolic content of *Citrus aurantium* juice was 295 ± 4 mg gallic acid equivalent(GAE)/g (fresh fruit extract) and the flavonoid content was 26 mg Quercetin-equivalent/g (Haraoui *et al*., 2020). In contrast, *Citrus aurantium* blooms contained only 81 ± 3 mg GAE/g extract and 20 ± 3 mg QE/g extract of total phenolic and flavonoids respectively (Değirmenci & Erkurt, 2020). The-fruit extract of *Citrus aurantium* has an important antioxidant potential and a higher level of total phenolic compounds compared to other species of the same gender such as *Citrus clementina*, *Citrus aurantifolia*, *Citrus hamlin, Citrus sinensis, Citrus limon*, *Citrus grandis*, and *Citrus nave* (Maksoud *et al*., 2021). A rich source of natural antioxidants is undoubtedly *Citrus aurantium*. Many extracts, including the leaves, peels, fruits, and pulp extract, showed evidence of its effect. IC 50 for the latter was 467 mol/g (Maksoud *et al*., 2021).

Secondly, we assessed the analgesic effect of *Citrus aurantium* extracts using two animal models. The hot plate test was employed to investigate the central mechanism, while the acetic acid-induced spasms test aimed to demonstrate the role of peripheral mechanisms. Our findings indicated that the juice extract at 200 mg/kg exhibited a more pronounced analgesic effect compared to the flower extract at the same dose. Nevertheless, both extracts demonstrated robust analgesic activity. It was observed that *Citrus aurantium* extracts increased the latency time in the hot plate test. This effect could be attributed to the action of their bioactive compounds on central opioid receptors or the activation of endorphins or enkephalins in the periaqueductal gray matter (Vuong *et al*., 2010). These endogenous peptides play a role in inhibiting the transmission of dopaminergic impulses at the dorsal horn synapse (Bhattacharya *et al*., 2016). In addition, in writhing test the extracts of *Citrus aurantium* decreased the number of spasms in mice which may be linked to the inhibition of certain chemical mediators such as serotonin, histamine and bradykinin that stimulate sensory nerve endings (Roh *et al*., 2020). These findings indicate that *Citrus aurantium* extracts have both central and peripheral actions. Our results concur with those of Khodabakhsh *et al*. (2015), who hypothesize that the essential oil of *Citrus aurantium* L. blossoms contains one or more physiologically active substances that have a central and peripheral antinociceptive impact.

Finally, the antilithiasic activity of *Citrus aurantium* extracts was revealed in the results of the current study. The juice extract exhibited a more pronounced antilithiasic effect compared to the flower extract in lithiasic rats. Following the oral administration of ethylene glycol 0.75% and ammonium chloride 2%, calcium oxalate stones grew and accumulated, forming crystal deposits in the renal tubules or pelvis. As the sedimentation rate increased, the size of the calcium oxalate stones also increased (Afzal *et al*., 2021). Urolithiasis was accelerated by ammonium chloride, which enhanced the effect of ethylene glycol (Azimi *et al*., 2021). The flower extract significantly restored the body weight of animals compared to lithiasic animals that experienced weight loss due to ethylene glycol consumption, confirming the effect of *Citrus aurantium* on weight restoration as established by Fugh-Berman & Myers (2004). This lithiasis model was validated by several previous research studies, including one reported by Hiremath & Jalalpure (2016). The increase in urea and creatinine levels is also employed as an indication of the presence of renal alterations in the lithiasis group. A reduction in the glomerular filtration rate induced by lithiasis blockage may lead to a buildup of nitrogenous compounds in the serum (Marhoume *et al*., 2021; Pawar & Vyawahare, 2017). According to our study, the flower and fruit extracts of *Citrus aurantium* most probably possess properties that prevent renal lithiasis, as they significantly decrease the levels of urea and creatinine. Similar results were observed by Sharma & Gilhotra (2022), who found that the hydroalcoholic extract of *Citrus aurantium* also reduced the levels of creatinine and uric acid, indicating that the use of this plant can interact with lithiasis.

The results of the histopathological studies were in line with the biochemical changes observed. *Citrus aurantium* extracts consistently prevented the potentially harmful changes to the renal tissue induced by the mixture of ethylene glycol and ammonium chloride. The preventive effects of *Citrus aurantium* extracts varied depending on the extract type. Both extracts at 200 mg/kg demonstrated a nephroprotective effect, but the fruit extract was the most effective in preventing the disruption of the renal architecture caused by calcium oxalate stones. Phenolic substances, including flavonoids, were identified for their ability to produce an antilithiasis effect (Brancalion *et al*., 2012). These substances also prevented crystal formation in vitro (Novaes *et al*., 2014). Terpenes' contribution to the prevention of calcium oxalate crystal aggregation has also been supported by numerous research (Afzal *et al*., 2021; Azimi *et al*., 2021). A significant amount of flavonoids and terpenes were found in *Citrus aurantium* fruit and flower extracts, which may account for their nephroprotective activity (Maksoud *et al*., 2021). The ability of *Citrus aurantium* extracts to act as antioxidants was suggested to be directly related to the observed antilithiasic characteristics following treatment. The production of papillary calculi is a consequence of intrapapillary calcifications induced by oxidative stress, a process that can be significantly reduced by antioxidants (Marhoume *et al*., 2021).

Citrus fruits, including *Citrus aurantium*, are rich sources of citrate, a significant inhibitor of urinary

stone formation. Citrate, naturally present in citrus fruit juices, has been demonstrated to elevate urinary citrate levels. This increase in urinary citrate levels is believed to have a favorable effect on the prevention of calcium stones (Cupisti *et al*., 2023; Lee, 2015). The presence of citrate in *Citrus aurantium* underscores its potential contribution to renal health, emphasizing the importance of exploring the interplay between *Citrus aurantium* extracts and the citrate content in the context of their effects on urinary stone formation.

Conclusion: In summary, our study underscores the significant antioxidant, analgesic, and antiurolithiasis activities associated with the oral administration of *Citrus aurantium* juice and fruit extracts. The observed effects present promising avenues for therapeutic applications. Nevertheless, further investigations are essential to pinpoint and develop the active compounds responsible for these beneficial properties, paving the way for the targeted and optimized utilization of these extracts in therapeutic interventions.

Conflict of interest: Authors declare no conflict of interest.

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Ethical issue: The European Community Guidelines (EEC Directive 86/609/EEC, dated November 24, 1986) were followed during the execution of all tests. There were several measures used to lessen the number of animals used and to minimize their suffering.

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