

Isolation, characterization and evaluation of antiurolithiatic phytoconstituents from *Moringa oleifera*

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Abstract

Moringa oleifera is a fast-growing tree, rich in secondary metabolites, and it grows in tropical and subtropical areas of the world. It is reported to treat more than 300 diseases. Kidney stone formation affects approximately 12% of the global population. Crude extracts of different parts of *M. oleifera* Lam. are reported to possess antiurolithiatic potential. This study aimed to explore the phytochemicals responsible for the antiurolithiatic property present in the seeds. In this research work, 30% aqueous methanolic seed extract was fractionated with non-polar to polar solvents using petroleum ether, dichloromethane, ethyl acetate, methanol, and water. Thin layer chromatography of fractions led to combine and subject to chromatograph the dichloromethane and ethyl acetate fractions over silica, which resulted in the isolation of two compounds characterized through UV, FT-IR, ¹H NMR, and ¹³C NMR. Furthermore, these compounds were evaluated for their antiurolithiatic potential, Niazimicin via *in vivo* and Niazimicin and Wajidinol via *in vitro* methods using Cystone, a herbal medicine, as a standard drug. Spectroscopic data helped to elucidate the compounds as Niazimicin, a thiocarbamate aromatic rhamnoside, while the second compound was thiocarbamate aromatic glucoside, named as Wajidinol. Both compounds inhibited CaOx nucleation, aggregation, and crystal growth, with Niazimicin showing greater activity. At the highest tested concentration, Niazimicin and Wajidinol, tested at 100-1000 µg/mL, inhibited nucleation, aggregation, and crystal growth by 65.6±3.1% and 54.9±0.8%, 75.2±0.5% and 57.2±3.4%, and 66.7±0.9% and 55.9±2.2% as compared to Cystone with the dose of 100-1000 µg/mL, which showed 69.3±2.3%, 80.4±0.3%, and 71.7±0.9%, respectively. While in experimental nephrolithiasis, Niazimicin further produced significant improvements in ethylene glycol-induced urolithiasis by reducing calcium oxalate crystalluria and improved in selected biochemical markers. The current research work clearly indicated that *M. oleifera* seeds contain secondary metabolites, consisting of thiocarbamate moieties, which demonstrated CaOx crystallization-modifying activity *in vitro* and preliminary activity against crystalluria *in vivo*. Further adequately powered studies are warranted to establish their therapeutic relevance in CaOx nephrolithiasis.

Keywords: Niazimicin, Wajidinol, *Moringa oleifera*, Antiurolithiatic, Spectroscopy, Kidney stone

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Introduction

Moringa oleifera Lam. (MO) belongs to the genus *Moringa* and family *Moringaceae*, which is native to tropical regions like India, Pakistan, and Africa. It has different regional names like marango tree, mulangay tree, saijna tree, suhanjna, horseradish tree, drumstick tree, benzolive tree, and kelor tree (Mahato et al., 2022; Matic et al., 2018). It grows well on poor soil, needs few inputs, and can withstand drought conditions, thus making it very suitable for sustainable agriculture in areas experiencing malnutrition, climatic changes, and soil erosion. It grows to 12 meters tall, usually in warm temperature (25-35°C) in direct or indirect sunlight and withstands the slightly acidic soil (Pareek et al., 2023). This plant is incorporated in different food categories like dairy products, food alternatives, meat production, beverages, salads, bakery items, and more, as this is a valuable source of proteins, minerals, vitamins, fiber, and fats (Ramesh et al., 2026; Zimazi et al., 2025). *M. oleifera* is reported to possess antioxidant (Llorent-Martínez et al., 2023), hypoglycemic (Yang et al., 2022), anticancer (Pappas et al., 2021), bactericidal (Li et al., 2023; Naeem et al., 2025), antiulcer (Masood et al., 2025), anticancer (Aliyu et al., 2026), anti-inflammatory (Zhygeldiyeva et al., 2025), hepatoprotective, renal protective, antihypertensive (Salam et al., 2025), antibacterial, antifungal (Kochorov et al., 2025), antidiabetic, anti-arthritic, antiglycation effects (Usman et al., 2025), and also used for the treatment of cardiovascular diseases (Shehzad et al., 2025; Ullah et al., 2025; Al-Ahmad et al., 2026; Naeem et al., 2025). *Moringa oleifera* leaves (MOL) are a potent source of nutrients, minerals, vitamins, proteins, and amino acids (Abd Rani et al., 2018). *Moringa oleifera* leaves (MOL) are a potent source of nutrients, minerals, vitamins, proteins, and amino acids (Abd Rani et al., 2018). Additionally, the plant has recently been reported to improve insulin sensitivity and reduce inflammation (Hegazy et al., 2025), improving bone regeneration (Hairi et al., 2025), and water treatment by removing methyl orange (Ikhlaq et al., 2025), enhancing the plant growth by mitigating the toxic effects of herbicides (Khan et al., 2025), and removing heavy metal ions (Orisawayi et al., 2026). Its carbon bases composites are recently reported to enhance the performance of the supercapacitors (Kumar et al., 2026). Different parts of *M. oleifera* exhibit diverse pharmacological properties, which justify its

traditional and modern medicinal applications (Asif et al., 2025; Kousar et al., 2025). The seeds, for instance, possess potent antioxidant and anticancer activities, which are of great interest due to their potential in neutralizing free radicals and inhibiting tumor growth (Abdull Razis et al., 2014; Ara et al., 2025). The pods, rich in essential nutrients and bioactive compounds, are traditionally utilized to combat malnutrition in lactating mothers, while also helping in the management of endocrine and digestive disorders (Liu et al., 2018). The bark contains alkaloids that exhibit ulcer-healing and cardiogenic effects, highlighting its importance in treating gastric and cardiovascular ailments (Choudhary et al., 2013). Moreover, the roots have been reported to alleviate toothache, thyroid imbalance, and gastrointestinal issues, likely due to their antimicrobial and anti-inflammatory constituents (Adejumo et al., 2012).

The growth of stones in the urinary tract is referred to as urolithiasis, a prevalent condition with a high recurrence rate worldwide (Hong et al., 2023). It is a global health disorder and is increasing day by day (Geraghty et al., 2023). Mainly, these stones are composed of oxalates or phosphates of calcium, uric acid, or cysteine. These stones are developed due to a variety of reasons, like dietary, environmental, or metabolic imbalance (Curhan et al., 2001). Urolithiasis affects between 7-13% of adults in North America, 5-9% of those in Europe, and 1-5% of those in Asia (Sorokin et al., 2017).

Different parts of *M. oleifera* are reported to possess antiurolithiatic potential in the form of crude extracts (Ali et al., 2024; Fahad et al., 2010; Karadi et al., 2006). This works via inhibition of calcium oxalate crystal inhibition, suppression of crystal growth, antioxidant potential and thus reducing oxidative renal injury, and improvement in hyperoxaluria-associated renal damage (Ali et al., 2024). However, despite numerous ethnomedicinal uses and biological activities of *Moringa oleifera*, the precise metabolites from its seeds responsible for the formation of CaOx stones have not been thoroughly investigated. Specifically, the impact of individual metabolites from the plant on the processes of CaOx nucleation, aggregation, and crystallization, as well as their connection to crystalluria, is understudied. That is why the aim of the present study is to purify and identify thiocarbamate glycosides from *M. oleifera* seeds, analyze their effect on various aspects of CaOx crystallization, and compare their activity with Cystone. Furthermore, the effect of niazimicin on

urinary crystalluria was evaluated in an ethylene glycol-induced animal model of CaOx crystalluria.

Material and Methods

Reagents, chemicals and instruments

For the phytochemical fractionation, solvents such as petroleum ether, dichloromethane, ethyl acetate, and methanol were distilled to remove impurities before use. The distilled solvents were also used to obtain extracts of varying polarity for further isolation of bioactive components through chromatography. Silica gel (mesh size 230-400, Sigma-Aldrich) was used as a stationary phase for column chromatography, enabling the separation of phytochemicals based on their polarity. Preparative thin-layer chromatography (TLC) cards (Silica gel-60F₂₅₄ deposited on an aluminum sheet) were used for checking the number and purity of components. Fluorescent tubes (254 and 366 nm) and 1% acidified aqueous solution of ceric (IV) ammonium sulfate were used for the visualization and location of compounds on the TLC plate. An Agilent Cary-630 ATR-FT-IR was used for infrared spectroscopic analysis, leading to the identification of functional groups in the purified compound. ¹H NMR and ¹³C NMR analysis was performed on a Bruker-500 MHz NMR spectrophotometer for structural establishment.

The various analytical-grade chemicals and instruments were used throughout the study to maintain accuracy and reproducibility in both *in vitro* and *in vivo* studies. Such as Cystone (Himalaya, India) has been used as a standard for evaluating antiurolithiatic efficacy. Ammonium chloride (Lahore Pharma, Pakistan), calcium chloride dihydrate, sodium carbonate, sodium chloride, sodium oxalate, and ethylene glycol (Merck, Germany) have been used for the induction of urolithiasis in experimental models. Anesthetic agents like ketamine (Global Pharmaceutical, Pakistan) and xylazine (My Lab, Pakistan) were used during *in vivo* procedures, minimizing animal distress. Sodium acetate trihydrate (Duksan, Korea), hydrochloric acid (BDH, England), ketamine (Budapest, Hungary), tris base (Fluka, USA), and formalin (Riedel-de Haen, Germany) were used for *in vitro* and *in vivo* studies. Biochemical parameters indicative of kidney function were analyzed quantitatively with the help of various analytical kits, such as calcium, sodium, potassium (Bio-active Diagnostic System, Germany), creatinine, magnesium, phosphorus, uric acid, urea, and total

protein (Human Diagnostic Worldwide, Germany) kits.

Plant collection

Moringa seeds (Figure 1) (voucher# CIDS/ IUB-3501/135) were collected from Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur (29.3544° N, 71.6911° E), Punjab, Pakistan, in fall 2020.



Figure-1. *Moringa oleifera* plant.

Extraction and isolation

The seeds were shade-dried and then ground into powder. Powder (1.5 kg) was macerated in aqueous methanol (3:7, v:v) for three days, followed by filtration and rotary evaporation, and this process was repeated thrice to obtain a thick, gummy mass weighing 41.5 g. The crude extract was fractionated *via* vacuum liquid chromatography (VLC) in increasing order of polarity using petroleum ether, dichloromethane (DCM), ethylacetate (EtOAc), methanol, and finally passed the distilled water giving aqueous fraction. The volume of each solvent used was 3L. The quantities obtained through this

fractionation were 60 mg, 1.0 g, 7.0 g, 21.0 g, and 8.7 g, respectively. Comparative thin-layer chromatography (TLC) of these fractions led to combine and chromatograph the DCM (D) and EtOAc (E) fractions whose gradient elution using petroleum ether, DCM, and ethyl acetate resulted in 15 sub-fractions (DEA-DEO). DEF (Niazimicin (NZ)) was identified as a pure compound weighing 5.55 g, while the second compound (Wajidinol (WJ)) was isolated from column chromatography of sub-fraction DEL. The purity of the compounds was checked with the help of TLC. Both of these compounds were found to be UV active at 254 nm, and characterized through Fourier-Transform Infrared spectroscopy (FT-IR) and proton nuclear magnetic resonance spectroscopy (^1H NMR) for structural elucidation. Also, the second compound was structurally elucidated through other NMR techniques such as ^{13}C NMR (Broad Band), distortionless enhancement by polarization transfer (DEPT-135), correlation spectroscopy (COSY), heteronuclear single quantum correlation spectroscopy (HSQC), and heteronuclear multiple bond correlation spectroscopy (HMBC) techniques, which helped to establish the structure.

Antiurolithiatic potential determination

In vitro studies

The effects of NZ and WJ on different phases of crystallization were investigated by crystal nucleation, aggregation, and growth assays. Experiments were conducted for three-observation trials for Cystone, NZ, and WJ, with mean and standard error of mean (SEM). These assays represent the physicochemical models of calcium oxalate (CaOx) and are employed to evaluate the crystallization-modifying effects rather than cellular mechanistic insights. The *in vitro* crystallization tests were done in three different experiments where all the experiments were carried out on different days under identical experimental conditions using freshly prepared materials. The data provided in the manuscript are the average $\pm$ SEM of these independent experiments ($n=3$).

Nucleation assay

In vitro crystal nucleation, aggregation, and crystal growth assays were carried out in triplicate using the technique reported earlier (Mosquera et al., 2020). By using a nucleation assay, the effects of compounds Niazimicin (NZ) and Wajidinol (WJ) on CaOx crystallization were investigated. In Tris-HCl (0.5 M)

and NaCl (0.15 M) buffer, at pH = 6.5, calcium chloride (0.005 M) and sodium oxalate (0.0075 M) solutions were prepared, while solutions of Cystone and compounds NZ and WJ were prepared in distilled water with different dilutions ranging 100-1000 $\mu\text{g/mL}$. One milliliter of each dilution of compounds and Cystone was combined with 3 mL of calcium chloride before the addition of 3 mL of sodium oxalate solution. These dilutions were heated in an oven for 30 minutes at 37 $^{\circ}\text{C}$ and then cooled to room temperature. Absorbance was recorded at 620 nm, and the percentage of inhibition of crystal nucleation was calculated using the equation 1 (Jamshed and Jabeen, 2022):

$$\text{Percent Inhibition} = [1 - (\text{OD}_{\text{Test}}/\text{OD}_{\text{Control}})] \times 100$$

Whereas, OD stands for optical density, test represents Cystone (standard) or test compound and control describes the negative control.

Aggregation assay

The effects of compounds on CaOx crystal aggregation were investigated. Separate 0.05 M solutions of calcium chloride and sodium oxalate were prepared. Then, both solutions were combined and heated in a water bath at 60 $^{\circ}\text{C}$ for an hour, and then incubated overnight in an oven at 37 $^{\circ}\text{C}$. 0.05 M Tris-HCl and 0.5 M NaCl buffer were used to prepare a CaOx crystal solution (80 mg/100 mL) at pH = 6.5. One milliliter of each of the compound and Cystone dilutions was vortexed with three milliliters of the CaOx solution. The mixture was incubated in the oven at 37 $^{\circ}\text{C}$ for 30 minutes. Absorbance was determined at 620 nm. The percent inhibition of aggregation was calculated using the nucleation assay formula as given above (Mosquera et al., 2020).

Crystal growth assay

The crystal growth assay was used to investigate how compounds affect crystal development. In distilled water, various dilutions of compounds and Cystone were prepared. In a 0.05 M sodium acetate buffer with a pH of 5.7, a CaOx slurry (3 g/2 mL) was prepared. At pH = 7.4, 1.5 mL of tris-HCl (0.01 M) and NaCl (0.09 M) solutions were combined with 1 mL of calcium chloride and 1 mL of sodium oxalate solutions (each 0.004 M). The addition of CaOx slurry (30 μL) followed by 1 mL of each dilution of compounds and Cystone in the reaction mixture, was observed for crystal formation. By monitoring the absorbance at

214 nm, the percent inhibition of crystal growth was computed as mentioned for the nucleation assay (Ali et al., 2024).

***In vivo* studies**

To assess the potential of NZ, an ethylene glycol-induced urolithiasis model was employed.

Experimental animals

Adult male Wistar albino rats (*Rattus norvegicus*) (150-270 g) were used in the study. Animals were maintained in an environment that comprised a temperature of 23-25 °C, a 12-hour light/dark cycle, regular food, and an libitum water supply (Farhan and Jabeen, 2022). All animals were maintained in the animal house of the Pharmacology Research Laboratory, Department of Pharmacology, Faculty of Pharmacy, The Islamia University of Bahawalpur, Pakistan. The Pharmacy Animal Ethics Committee (PAEC) approved the experimental methods and protocols with reference number PAEC/24/109. The experimental animals were handled according to the local and international ARRIVE guidelines.

Antiurolithiatic potential of Niazimicin

To assess the antiurolithiatic efficacy of Niazimicin (NZ), albino rats were treated with drinking water mixed with 1% ammonium chloride (AC) and 0.75% ethylene glycol (EG) during the first five days and thereafter with EG for 16 more days to cause calcium oxalate (CaOx) urinary crystals. In the liver, EG gets absorbed and metabolized to glycolic acid, which is oxidized further to glyoxylic acid and yields oxalic acid by glycolate oxidase (Makasana et al., 2014). As the final product, oxalic acid complexes with free calcium and leads to calcium oxalate crystal deposition. Microscopic examination of the urine was initially conducted to view the kidneys' crystals in urine and compare the findings of EG-treated animals with those of the normal control group (Jamshed and Jabeen, 2022).

Animal model of urolithiasis

Animals were divided into six groups, each consisting of four rats, as natural products are obtained in smaller amounts, and thus, the number of rats per group was maintained at four. The current *in vivo* experiment was designed as a pilot/exploratory study to evaluate the antiurolithiatic potential of NZ, under conditions of limited availability of the compound. A fixed group

size of n=4 per group was selected in alignment with ethical considerations to minimize the animal rule, while allowing the obtaining of meaningful results. One group, which received tap water, was regarded as the normal control group, and the other groups underwent lithogenic treatment for the first 21 days and were considered as lithogenic groups. The relevant therapies were given for the following 14 days after the lithogenic treatment. Distilled water (5 mL/kg p.o.) was given to the normal control and intoxicated groups. Cystone (500 mg/kg p.o.) was given to the standard group once per 24 hours. The other groups, however, received various doses of NZ, such as 50, 100, and 150 mg/kg p.o., once every 24 hours, respectively (Ali et al., 2024).

Urine collection and analysis

Animals were put into the metabolic cages for urine collection at the end of the 21st, 28th and 35th day. 1 mL of fresh urine samples was taken in the morning and centrifuged for 5 minutes at 3000 rpm for the count of crystals. The supernatant (950 µL) was removed, and the rest was put on a Neubauer chamber. The crystal types and number were detected with a light microscope as aforementioned (Khan et al., 2011). 24 hours later, urine was collected, and urinary volume, pH, uric acid, magnesium, phosphorus, and total protein were measured with the aid of commercial kits. On the 21st and 35th day, animals were anesthetized with ketamine/xylazine (10:1), and blood samples were collected by retro-orbital and cardiac puncture methods. The retro-orbital way was adapted on the 21st day of the experiment to maintain the survival of the animals, while for the final test on the 35th day, blood samples were collected through cardiac puncture under deep anesthesia as a terminal procedure. Following blood collection, animals were euthanized by cervical dislocation to ensure death. Fifteen minutes later, blood samples were centrifuged at 4000 rpm for 15 minutes (Jamshed and Jabeen, 2021). Then, sera were analyzed for the estimation of biochemical parameters, i.e., calcium, creatinine, blood urea nitrogen (BUN), sodium, and potassium, using commercially available kits.

Urinary crystalluria and related parameters were measured repeatedly in each animal of each group at multiple times, i.e. 21st, 28th and 35th day, rather than using mixed-effects models. For these longitudinal outcomes, statistical comparisons were performed using two-way ANOVA with treatment and time as factors, followed by Bonferroni's post hoc test.

Post hoc power analysis

A post hoc power analysis was performed to assess the adequacy of the sample size during this *in vivo* study. Power calculations were performed based on measured urinary crystal counts and serum levels, showing the treatment effects. This analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test, with a significance level (α) of 0.05. Effect sizes (Cohen's f) were calculated from the observed group means and standard deviations obtained in ethylene glycol-induced urolithiasis. The observed effect sizes ranged from 0.45 to 0.70, revealing the robust treatment-related differences between intoxicated, control, and treated groups. Based upon the effect sizes and sample size of animals per group ($n=4$), the achieved statistical power ($1-\beta$) ranged from 0.80 to 0.92, indicating that the study was sufficiently powered to detect the biologically meaningful differences despite the limited group size. The sample size was also aligned with the 3Rs principle (Replacement, Reduction, Refinement) to minimize animal use while maintaining statistical power.

Results and Discussion

Characterization of Niazimicin

Appearance: gummy sticky material. Quantity: 5.55 g (13.2% w/w to extract, 0.37% to dry powder). FT-IR (ZnSe, $\nu=\text{cm}^{-1}$): 3310.8 (O-H) (br, alcohol), 2935 (C-

H)(Ar-H), 1610 (C=C)(olefinic), 1507 (H-C-H)(bending), 1226 (C-O) (ether and alcohol), 1120 (C=S)(thiocarbamate), 1058 (C=S)(thiocarbamate). ^1H NMR (500MHz, CD_3OD , $\delta=\text{ppm}$): 1.23 (d, 3H, $\text{CH}_3\text{-CH-C}$), 1.24 (t, 3H, $\text{CH}_3\text{-CH}_2\text{-O}$), 3.47 (m, 1H, CH-O), 3.64 (m, 1H, CH-O), 3.85 (m, 1H, CH-O), 4.01 (m, 1H, CH-O), 4.36 (s, 1H, N-H), 4.65 (s, 2H, Ar- $\text{CH}_2\text{-N}$), 5.42 (d, 1H, 2Hz, O- CH-O), 5.45 (d, 1H, 2Hz, O- CH-O), 7.07 (dd, 2H, 8Hz, Ar-H), 7.29 (dd, 2H, 8Hz, Ar-H).

Niazimicin was isolated and purified from hydromethanolic seed extract through column chromatography using a mobile phase of 30-50% EtOAc in DCM. This compound was a major constituent (5.55 g, 12.9% yield to extract and 0.38% yield to dried powder) of MO and was obtained as a viscous liquid that was soluble in either DCM, methanol, or water. FT-IR data (Figure 2) indicated the presence of many functionalities. Such as a broad absorption signal at 3310 cm^{-1} indicated the presence of either a hydroxyl group or an amino group (Huang et al., 2023). A sharp signal at 2935 cm^{-1} indicated C-H stretching, while a signal at 1610 cm^{-1} showed the presence of an olefinic bond (Nandiyanto et al., 2019). A strong absorption signal at 1507 cm^{-1} indicates CH_2 bending vibration, whereas 1226 cm^{-1} indicates C-O stretching vibration. The existence of thiocarbamate and C=S functionalities in the molecule was revealed by vibrations at 1120 and 1058 cm^{-1} , respectively (Xue et al., 2019).

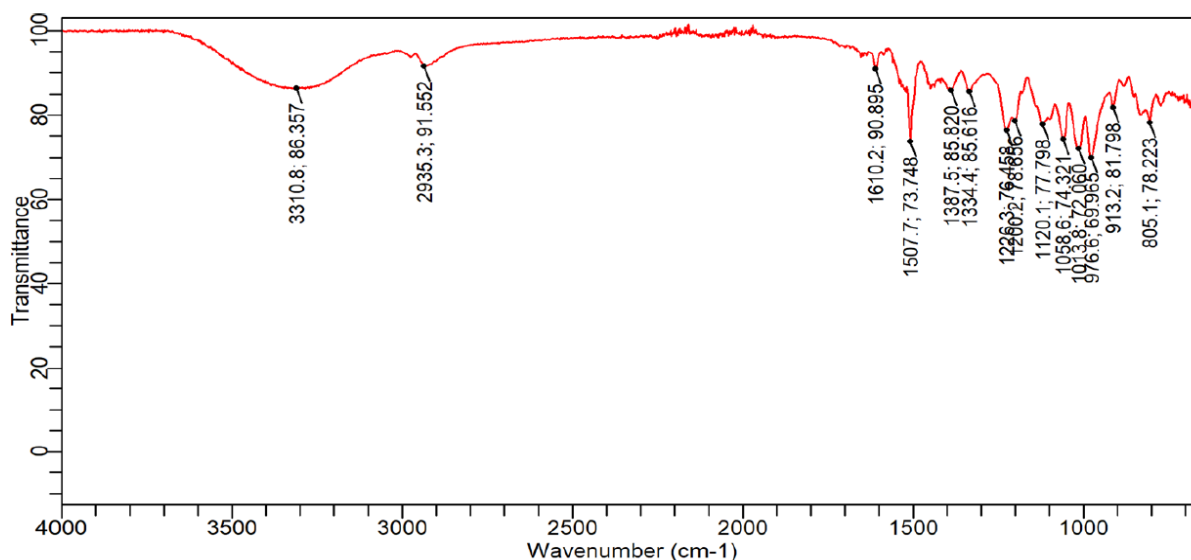


Figure-2. FTIR graph of compound Niazimicin.

^1H NMR data indicated two methyl groups at δ 1.23 and 1.24 ppm resonated as a doublet and a triplet, respectively. The presence of a deshielded methylene protons' singlet at δ 4.65 ppm indicated the benzylic moiety (Ar-CH_2 -) in the system. Two doublets appearing at δ 5.42 and 5.45 ppm of H1' revealed the nature of the proton to be anomeric ($-\text{O-CH-O}$) and thus showing the compound to be glycosidic, while these doublets with 1:2 ratio indicated the isomeric (thiol-imine tautomeric) nature of the compound, and their J -value of 2 Hz indicated that the anomeric proton was oriented to the equatorial position. A signal of one proton appeared as a singlet at δ 4.36 ppm and was considered for the amino proton ($-\text{NH}-$). Since the compound exists as a thiol-imine tautomeric form, this upfield signal indicated that the amino proton is *anti*-

to the sulfur (as shown below in figure 2), while its *syn*- signal appeared at δ 9.89 ppm. Furthermore, two doublets of doublet at δ 7.07 (H2 and H6) and 7.29 ppm (H3 and H5) with a J -value of 8.0 Hz indicated the *para*-substituted compound with *ortho*-coupling of the aromatic protons (Ar-H). Multiplets of single protons each appeared at δ 4.01, 3.85, 3.47, and 3.64 ppm, indicating the oxygenated carbon system (O-CH-) of glycosidic moiety and were assigned to H2' , H3' , H4' , and H5' , respectively.

All the spectroscopic data was correlated to the reported data of NZ, and hence, the isolated compound was deduced as *o*-ethyl-4-[(α -L-rhamnosyloxy)benzyl]-thiocarbamate or Niazimicin (NZ) (Figure 3) (Abdelsayed et al., 2021).

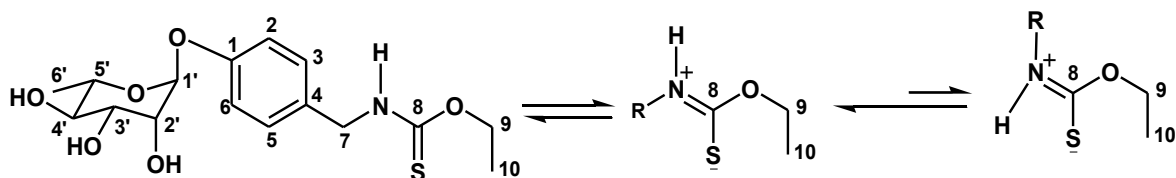


Figure-3. Structure of Niazimicin interpreted through spectral data.

Characterization of Wajidinol

Appearance: Gummy material. Quantity: 37 mg (0.08% w/w to extract). FTIR (ZnSe , $\nu=\text{cm}^{-1}$): 3322 (O-H) (br, alcohol), 2931 (C-H)(Ar-H), 1610 (C=C)(olefinic), 1507 (H-C-H)(bending), 1228 (C-O) (ether and alcohol), 1120 (C=S)(thiocarbamate), 1058 (C=S)(thiocarbamate). ^1H NMR (500MHz, CD_3OD , $\delta=\text{ppm}$): 1.24 (t, 3H, $\text{CH}_3\text{-CH}_2\text{-O}$), 3.48 (m, 1H, CH-O), 3.64 (m, 1H, CH-O), 3.86 (m, 1H, CH-O), 3.99 (m, 1H, CH-O), 4.36 (s, 1H, N-H), 4.65 (s, 2H, $\text{Ar-CH}_2\text{-N}$), 5.42 (d, 1H, 2Hz, O-CH-O), 5.45 (d, 1H, 2Hz, O-CH-O), 7.08 (dd, 2H, 8Hz, Ar-H), 7.28 (dd, 2H, 8Hz, Ar-H). ^{13}C NMR (125MHz, CD_3OD , $\delta=\text{ppm}$): 16.6 (1C, $\text{CH}_3\text{-CH}_2\text{-O}$), 45.4 (1C, $\text{Ar-CH}_2\text{-N}$), 55.9 (1C, $\text{O-CH}_2\text{-CH}_3$), 69.2 (1C, O-CH-CH-O), 69.3 (1C, O-CH-CH-O), 70.6 (1C, O-CH-CH-O), 70.8 (1C, O-CH-CH-O), 72.4 (1C, O-CH-CH-O), 98.5 (1C, O-CH-O), 116.1 (1C, Ar-C), 116.7 (1C, Ar-C), 128.7 (1C, Ar-C),

128.9 (1C, Ar-C), 131.6 (1C, Ar-C), 156.0 (1C, Ar-C), 191.8 (1C, N-C(=S)-O).

Wajidinol was isolated from hydromethanolic seed extract through column chromatography eluted at 55-70% methanol in DCM, apparently as gummy material, and showed its solubility in either methanol or water. FT-IR data (Figure 4) indicated various functionalities present in the compound. The presence of a hydroxyl group was observed by the broad absorption signal at 3322 cm^{-1} in the FT-IR spectrum (Huang et al., 2023). At 2931 cm^{-1} , C-H stretching was observed. A sharp signal at 1610 cm^{-1} indicated the presence of the C=C olefinic bond, and CH_2 bending vibrations were detected at 1507 cm^{-1} (Huang et al., 2023). A signal indicating the existence of C-O was observed at 1228 cm^{-1} . Thiocarbamate and C=S vibrations were detected at 1120 and 1058 cm^{-1} (Xue et al., 2019).

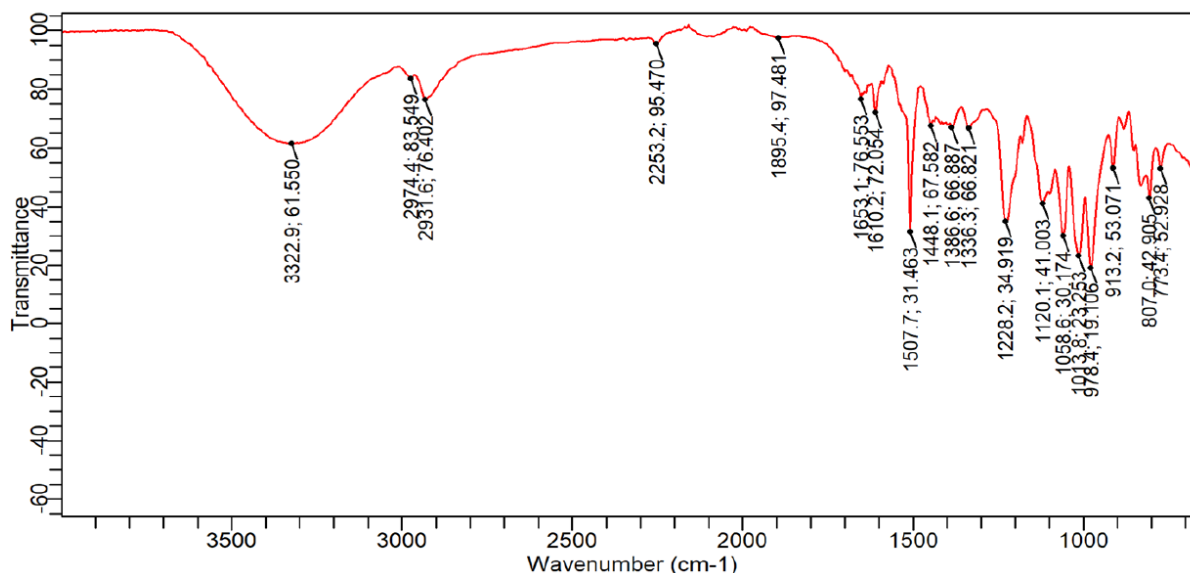


Figure-4. FTIR graph of compound Wajidinol.

^1H NMR data showed a triplet of three protons appeared at δ 1.24 ppm (H10), indicating an ethyl ($\text{CH}_3\text{-CH}_2\text{-}$) fragment present in the molecule. The singlet of one proton at δ 4.36 ppm was considered to belong to the amino group (-NH). Another singlet of two protons, *i.e.*, methylene, appeared at δ 4.65 ppm (H7), indicating its attachment to the aromatic system and to be benzylic in nature ($\text{Ar-CH}_2\text{-}$). Two doublets appeared at δ 5.42 and 5.45 ppm, with coupling constants of 2 Hz each, revealing the anomeric nature (H1') of one proton each (-O-CH-O-) with a 1:2 ratio showing the isomeric (thiol-imine tautomers) nature of the molecule, and a coupling constant of 2.0 Hz indicated the position of the proton to be equatorial. Since the compound has thiocarbamate functionality, it is considered to exist as thiol-imine tautomers, whose -NH signal in the *E*-tautomeric form appears upfield at δ 4.36 ppm while that of the *Z*-isomer appears at δ 9.89 ppm. A couple of doublet of doublet appeared at δ 7.08 (H2 and H6) and 7.28 ppm (H3 and H5) of two protons each with a *J*-value of 8.0 Hz indicated para substitution at the benzene ring (Ar-H). Four sets of multiplets appeared at δ 3.48, 3.64, 3.86, and 3.99 ppm, indicating the glycosidic nature of protons attached to oxygenated carbon atoms (-O-CH-), and these values were assigned to H4', H5', H3', and H2', respectively. This information led to the conclusion that the compound in hand is comprised of a benzene system with 1,4-substitution, and it is

benzylic, with a glucosidal attachment and *O*-ethyl thiocarbamate fragments.

^{13}C NMR data with DEPT-135 analysis helped to count the number and nature of carbon atoms. This data showed 16 carbon atoms, among those one methyl, three methylenes, nine methines, and three carbon atoms with no hydrogen were present. A signal appeared at δ 191.8 ppm, indicating the carboxyl (thiocarbamate) nature of carbon. Six carbon signals appeared as olefinic (aromatic) in nature, ranging from δ 116.1 to 156.0 ppm. The most downfield signal appeared at δ 156.0 ppm among these six signals and was assigned to C1 as it was directly attached to an electron-withdrawing group, *i.e.*, oxygenated system. Most upfield signals, *i.e.*, δ 116.1 and 116.7 ppm, were assigned to C2 and C6 as adjacent to oxygenated carbon, and the positive resonance effect shielded these carbon centers. Signals appeared at δ 128.7 and 128.9 ppm and were assigned to C3 and C5, whereas the δ 131.6 ppm value was assigned to C4 as it was attached to a non-aromatic carbon atom that was further attached to the thiocarbamate functionality. One of the characteristic peaks appeared at δ 98.5 ppm, indicating the anomeric nature of carbon, and hence, there might be the presence of a sugar moiety in the molecule. Signals appeared at δ 72.4, 70.8, 69.2, 69.3, and 70.6 ppm belonging to the glycosidic part of the molecule and assigned to C2', C3', C4', C5', and C6', respectively. The signal that appeared at δ 45.4 ppm was assigned to benzylic methylene (C7), while the

signal that was observed at δ 16.6 ppm was that of the methyl group (C10).

Our previously discussed compound, Niazimicin, showed a merged doublet and triplet of two methyl protons at δ 1.23 and 1.24 ppm, while this compound showed only a triplet of methyl protons at δ 1.24 ppm, which indicated the presence of a glucoside moiety instead of a rhamnoside as in the case of NZ

(Abdelsayed et al., 2021). This difference led us to conclude the isolation of a new metabolite from MO seeds whose systematic name can be written as *o*-ethyl-4-(3,4,5-trihydroxy-6-(hydroxymethyl)-tetrahydro-2*H*-pyran-2-yl-oxy) benzylcarbamothioate, and its trivial name is given as Wajidinol (WJ). The structure of Wajidinol is shown below (Figure 5).

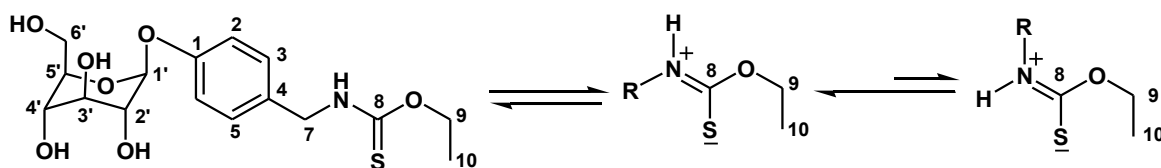


Figure-5. Structure of Wajidinol interpreted through spectral data.

2D-NMR techniques further helped to confirm the structure of the WJ unambiguously. The ^1H - ^1H COSY spectrum showed the hydrogen-hydrogen correlation present at adjacent positions. Spectrum showed the correlation of the aromatic proton of position 2 (δ 7.08 ppm) with that of position 3 (δ 7.28 ppm). Also, H9 appeared at δ 4.02 ppm coupled with H10, appearing at δ 1.24 ppm. In the glucosidic moiety, anomeric proton H1' with chemical shift of δ 5.42 ppm, coupled

with proton present at position 2' with chemical shift value of δ 3.99 ppm, which further coupled with H3' appeared at δ 3.86 ppm. H3' at δ 3.86 ppm and H4' at δ 3.48 ppm are coupled with each other, while H4' is further coupled with H5' at δ 3.64 ppm. H5' appeared at δ 3.64 ppm, coupled with the hydrogen atoms of position 6' that appeared at δ 3.96 and 4.01 ppm. These correlations are shown below (Figure 6).

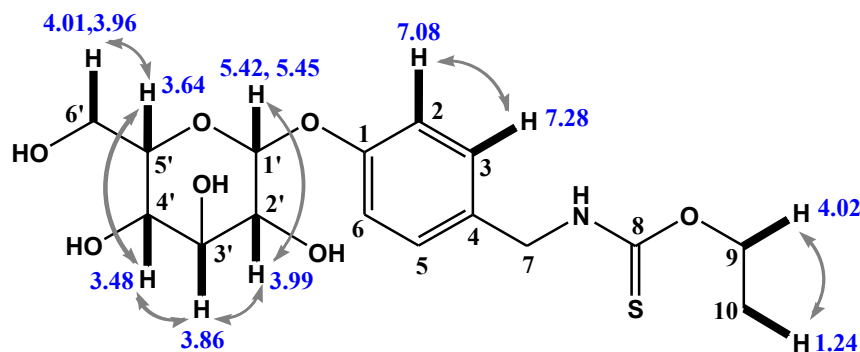


Figure-6. COSY of Wajidinol showing coupling of ^1H nuclei with their vicinal ^1H nuclei.

Also, heteronuclear single quantum correlation (HSQC) indicated the attachment of hydrogen to its parent carbon. The HSQC spectrum showed the coupling of proton H2 at δ 7.08 ppm with carbon C2 at δ 116.1 ppm. Hydrogen H3 with a chemical shift of δ 7.28 ppm, coupled with carbon C3 having a chemical shift of δ 128.7 ppm. Benzylic methylene also showed HSQC coupling as H7 appeared at δ 4.65 ppm coupled with C7 δ 45.4 ppm. The *o*-ethyl group also showed H-C correlations as H9 (δ 4.02 ppm) coupled with C9

(δ 55.9 ppm) and H10 (δ 1.24 ppm) coupled with C10 (δ 16.6 ppm). In the glucosidic part, H1' with a chemical shift of δ 5.42 and 5.45 ppm was observed to couple with C1', showing a chemical shift value of δ 98.5 ppm. H2' and H3' with chemical shifts of δ 3.99 and 3.86 ppm coupled with their respective carbon centers C2' and C3', having chemical shifts of δ 72.4 and 70.8 ppm, respectively. Similarly, H4' (δ 3.48 ppm), H5' (δ 3.64 ppm), and H6' (δ 3.96 and 4.01 ppm) showed HSQC correlations with C4' (δ 69.2

ppm), C5' (δ 69.3 ppm), and C6' (δ 70.6 ppm), respectively. These correlations are shown in the following figure (Figure 7).

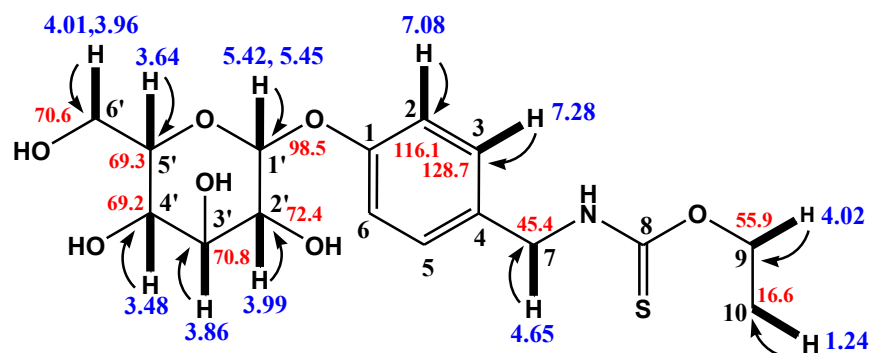


Figure-7. HSQC of Wajidinol showing coupling of ^1H nuclei with their parent ^{13}C nuclei.

Heteronuclear multiple bond correlation spectroscopy (HMBC) is helpful for intramolecular connectivities and structural establishment by revealing the information of hydrogen coupling with nearby carbon atoms except the parent carbon. HMBC spectrum indicated the long-distance coupling of the *o*-ethyl group correlating H9 (δ 4.02 ppm) with C10 (δ 16.6 ppm) and that of H10 (δ 1.24 ppm) with C9 (δ 55.9 ppm). Amino proton, N-H, appeared at δ 4.36 ppm, also coupled with C7 and C8, which appeared at δ 45.4 and 191.8 ppm, respectively. In the aromatic part, H2, having a chemical shift value of δ 7.08 ppm was

observed to couple with C1, C3, and C4, having chemical shifts of δ 156.0, 128.7, and 131.6 ppm, respectively. H3 (δ 7.28 ppm) coupled with C2 (δ 116.1 ppm) and C4 (δ 131.6 ppm). Benzylic proton H7 appeared at δ 4.65 ppm and was observed to couple with C8, which appeared at δ 191.8 ppm. In the glycon part, H1' that appeared as a pair of doublets at δ 5.42 and 5.45 ppm coupled with C1 and C2' appeared at δ 156.0 and 72.4 ppm, respectively. Both H4' (δ 3.48 ppm) and H6' (δ 3.96 and 4.01 ppm) coupled with C5' (δ 69.3 ppm). These HMBC correlations of WJ are shown in the following figure (Figure 8).

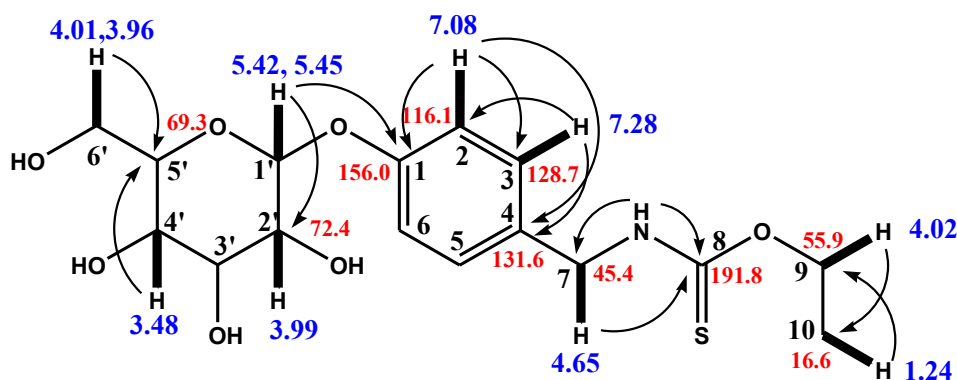


Figure-8. HMBC of Wajidinol showing long-range ^1H and ^{13}C coupling.

The present study was targeted to explore the phytochemistry of the hydromethanolic seeds' extract of *Moringa oleifera* responsible for antiurolithiatic potential. Different parts of the plant are already reported for this property in crude extract forms, however, no work has been published earlier to correlate phytochemicals with respective biological activity. Therefore, the seeds (1.5 kg) were ground into powder form, followed by maceration in 30% aqueous methanol for three days. Subsequently, it was filtered, and the filtrate was evaporated to get a thick, gummy material (41.5 g). This crude extract was subjected to VLC with gradient elution, using 3L of each solvent, such as petroleum ether, dichloromethane (DCM), ethyl acetate, methanol, and finally distilled water. The hydromethanolic seeds' extract of *M. oleifera* yielded a petroleum ether fraction in small quantity (60 mg, 0.14% of the total extract). Similarly, the DCM fraction of the extract was found 1.0 g (2.41%), ethyl acetate gave 7.0 g (16.83%), methanol afforded 21.0 g (50.60%), and distilled water furnished 8.7g (20.96%). Among those, DCM and ethyl acetate fractions were pooled together based on their comparative TLC. Column chromatography of these combined fractions led to the isolation and purification of secondary metabolites. Two compounds were isolated and characterized as Niazimicin and Wajidinol. Niazimicin, a thiocarbamate mainly found in the seeds, was isolated as a major metabolite 5.55g, which is 13% yield to crude extract, and 0.37% yield to dry powder. Also, the second compound, *i.e.*, Wajidinol, a glucose isomer of Niazimicin, was obtained in 37 mg quantity, which is only 0.08% yield of crude extract. These compounds were characterized through spectroscopic techniques such as UV, FT-IR,

and 1D- and 2D-NMR, to establish the structures of these purified compounds, and based on extensive literature survey and databases, it led us to conclude that the Wajidinol is a new compound isolated from any natural resource, to the best of our knowledge, and not previously reported from any source.

These isolated compounds were evaluated for their antiurolithiatic potential. Presumably, these phytochemicals might be good candidates for the treatment of kidney stones. Previously, Niazimicin has been found to possess spasmolytic activity (Gilani et al., 1994) and is a candidate for Medical Expulsive Therapy (MET); therefore, it can also be proven to be good in the management of urolithiasis.

Antiurolithiatic potential of Niazimicin and Wajidinol

In vitro studies

The percent inhibition of nucleation of NZ and WJ, at the highest concentration, was found to be 65.6 ± 3.1 and $54.9 \pm 0.8\%$, respectively, as compared to Cystone, which showed $69.3 \pm 2.3\%$ inhibition. The percent inhibition in the aggregation assay for Cystone was $80.4 \pm 0.3\%$, whereas NZ and WJ showed percent inhibitions of $75.2 \pm 0.5\%$ and $57.2 \pm 3.4\%$, respectively, at the concentration of 1000 $\mu\text{g/mL}$. The percent reduction in crystal growth for Cystone was calculated as $71.7 \pm 0.9\%$ at 1000 $\mu\text{g/mL}$, compared to NZ ($66.7 \pm 0.9\%$) and WJ ($55.7 \pm 0.9\%$). A graphical representation of the results over different concentrations is illustrated below in Figure 9. The results are expressed here with mean $\pm$ SEM of 3 observations of each group.

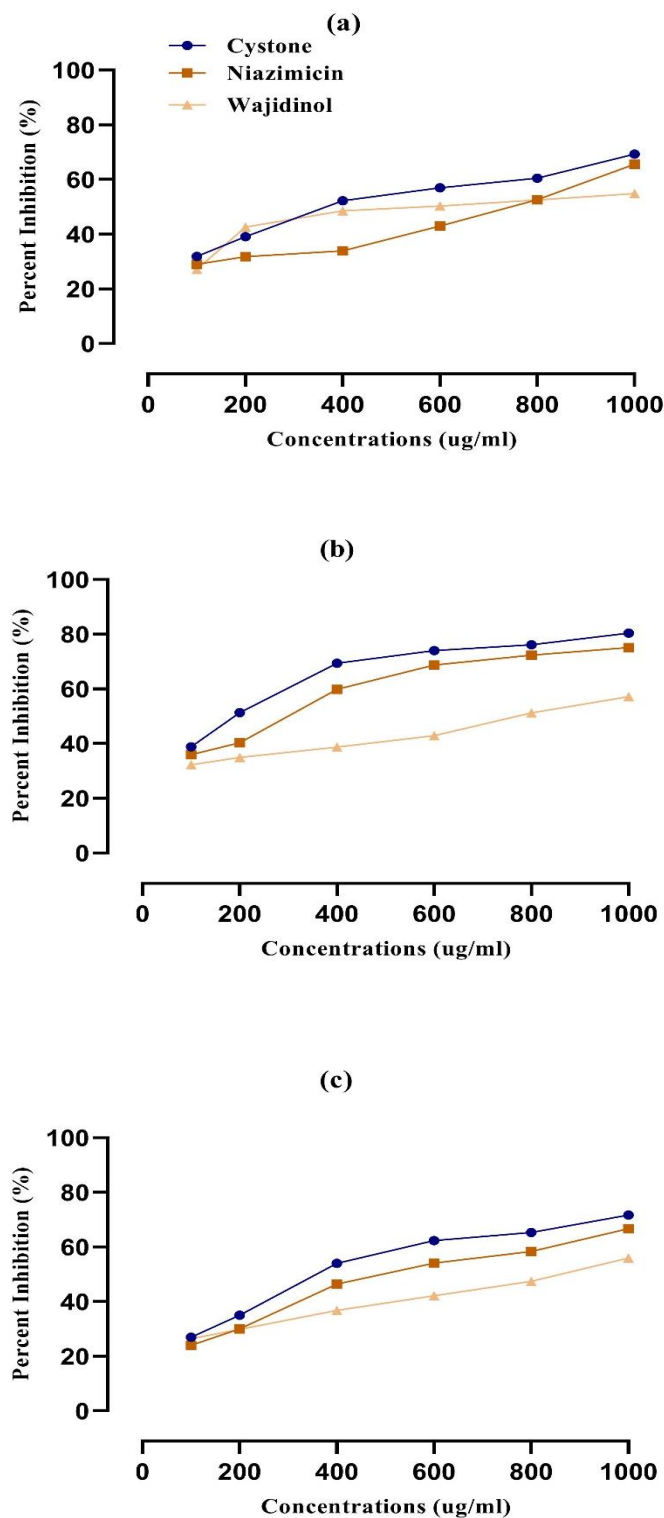


Figure-9. The effects of Cystone, NZ, and WJ on (a) nucleation, (b) aggregation, and (c) crystal growth. The values are expressed as Mean $\pm$ SEM of 3 observations in each group (Mean $\pm$ SEM; n=3).

In an *in vitro* model, both of our purified compounds (Niazimicin and Wajidinol) exhibited prevention of calcium oxalate (CaOx) crystallization processes such as nucleation, aggregation, and growth. Supersaturation-induced nucleation involves spontaneous crystallization, which was reduced by employing NZ and WJ by producing complexes with free calcium and oxalate ions, hence blocking crystal formation (Mosquera et al., 2020). CaOx's polymorphic forms, calcium oxalate monohydrate (COM) and dihydrate (COD), alter crystal behavior, with COM being more susceptible to urothelium binding and aggregation. Healthy urine contains anionic macromolecules such as glycosaminoglycan, which adhere to positively charged COM surfaces and reduce epithelial cell attachment via charge repulsion (Jamshed and Jabeen, 2022). Crystal aggregation, which is vital to stone formation, is more apparent in kidney stone patients than in healthy persons (Sheng et al., 2005; Wesson and Ward, 2006). The present research demonstrated the strong effects against crystal growth and aggregation in a dose-dependent manner, perhaps because of the capacity to form complexes with calcium and oxalate ions.

Antiuro lithic activity, crystal-modifying effects, and possible mechanisms of action can all be quickly evaluated using an *in vitro* experiment. As these assays are non-cellular, the observed effects are based on direct modulation of calcium oxalate crystallization. However, due to the complexity of the biological systems involved and the pathophysiology of urolithiasis, the results of these assays cannot be safely or directly summarized to therapy outcomes. Thus, to better understand kidney stone formation and assess the antiuro lithic potential, *in vivo* urolithiasis models

were used. Both of the compounds showed good efficacy against urolithiasis via *in vitro* analysis, but due to a higher quantity, only the Niazimicin was subjected to *in vivo* evaluation for the antiuro lithic potency.

***In vivo* studies**

Effects of Niazimicin on urinary parameters

Urine parameters, such as crystal count, urinary volume, and pH, uric acid, magnesium, phosphorus, and total protein levels were measured on days 21, 28, and 35 of the study, and effects of 50, 100, and 150 mg/kg doses of Niazimicin on these parameters were investigated, the findings are summarized in Figure 10. The primary endpoint of the study was urinary CaOx crystalluria, while the secondary endpoint comprised of sera and other biochemical parameters. Large effect sizes (Cohen's $f > 0.40$) were obtained based on observed group means and variability, supporting the treatment-related trends despite of the small sample size.

Results illustrate that NZ afforded highly significant and dose-dependent effects. At the doses of 100 and 150 mg/kg, NZ results were nearly identical to those of Cystone on the 28th day of sample collection, but significantly better on the 35th day (Figure 10a). Excessive and enormous crystals were excreted by intoxicated rats as compared to the normal control group. NZ, at the dose of 50 mg/kg, did not show any significant reduction in urine volume, but at the doses of 100 and 150 mg/kg, showed highly significant results ($p < 0.001$) and reduced the urine volume (Figure 10b) as compared to the normal control group.

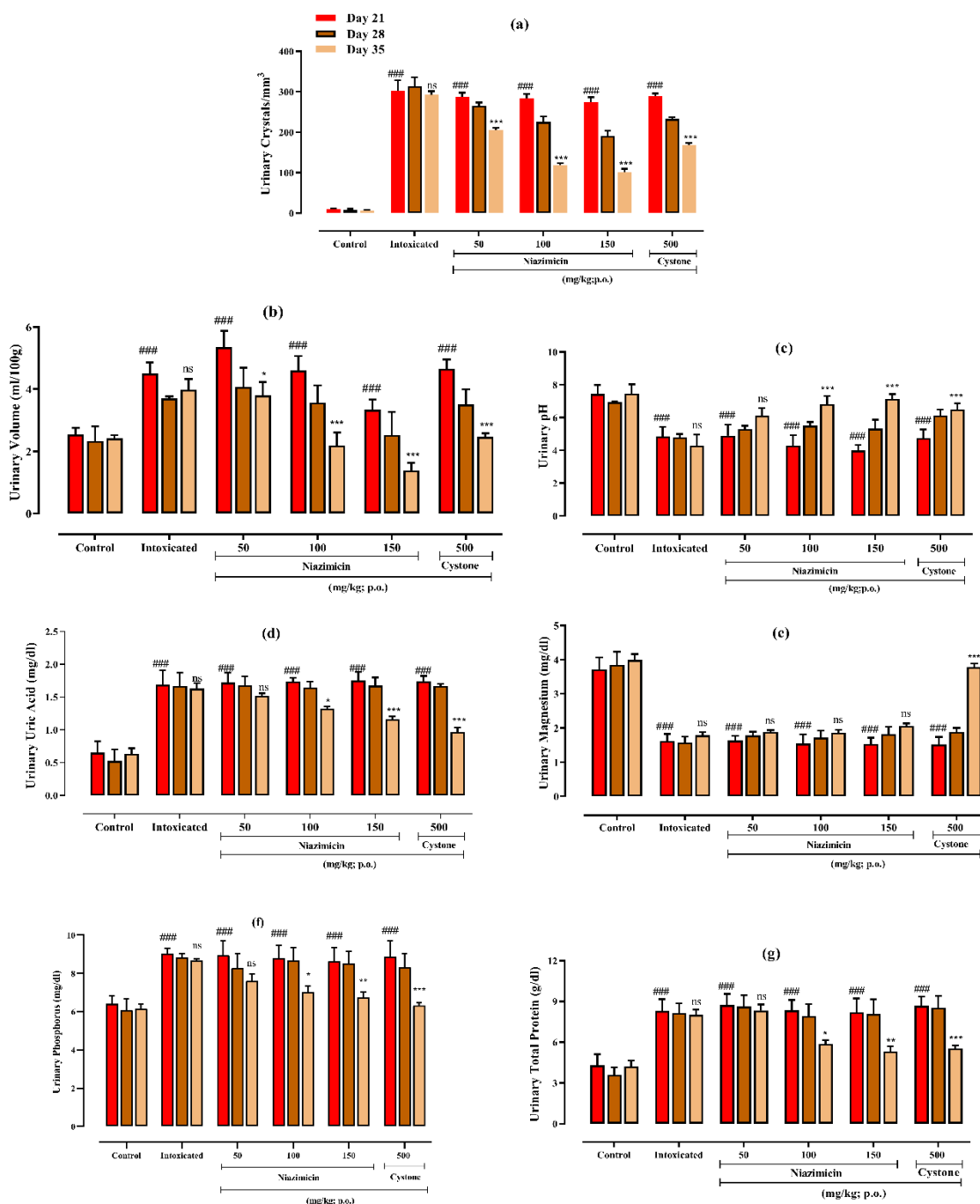


Figure-10. The effects of NZ and Cystone on urinary parameters (Mean $\pm$ SEM; $n=4$), two-way ANOVA followed by Bonferroni's post hoc test. Level of significance: ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs intoxication after 21 days and ###: $p < 0.001$ vs control group.

Ammonium chloride (AC), which was used in lithogenic treatment, lowered urine pH and promoted the deposition of CaOx crystals in the intoxicated groups (Figure 10). NZ raised the urinary pH in a dose-dependent manner on all sample collection days, but

particularly on the 35th day, demonstrating highly significant results. At the doses of 50, 100, and 150 mg/kg p.o., NZ significantly reversed urine pH levels to normal, and its effects were nearly identical to those of Cystone, the standard drug (Figure 10c). Lithogenic

treatment caused significant elevation ($p < 0.001$) in urinary uric acid, phosphorus, and total protein levels after 21 days. Whereas, NZ showed significant results at the doses of 100 and 150 mg/kg and reversed these parameters towards normal (Figure 10d, 10f, and 10g). Lithogenic treatment significantly reduced the magnesium levels after 21 days, NZ slightly restored its levels but not to a significant extent (Figure 10e).

Effects of NZ on serum parameters

Lithogenic treatment after 21 days (Figure 11) significantly increased serum creatinine and blood urea nitrogen (BUN) levels as compared to the normal

control group (Figure 11a and 11b). NZ showed highly significant results at 150 mg/kg and restored these parameters towards normal. Increased serum calcium levels ($p < 0.001$) were observed in lithogenic rats due to the deposition of crystalline material, specifically CaOx (Figure 11c). These elevated levels were attenuated by treatment with NZ, which shows significant results at the dose of 150 mg/kg. As depicted in figure (11d and 11e), serum sodium and potassium levels were significantly higher in the lithogenic group as compared to the normal control group. Treatment with NZ showed a significant decrease in these levels, and the highest dose (150 mg/kg) was found to be the most effective.

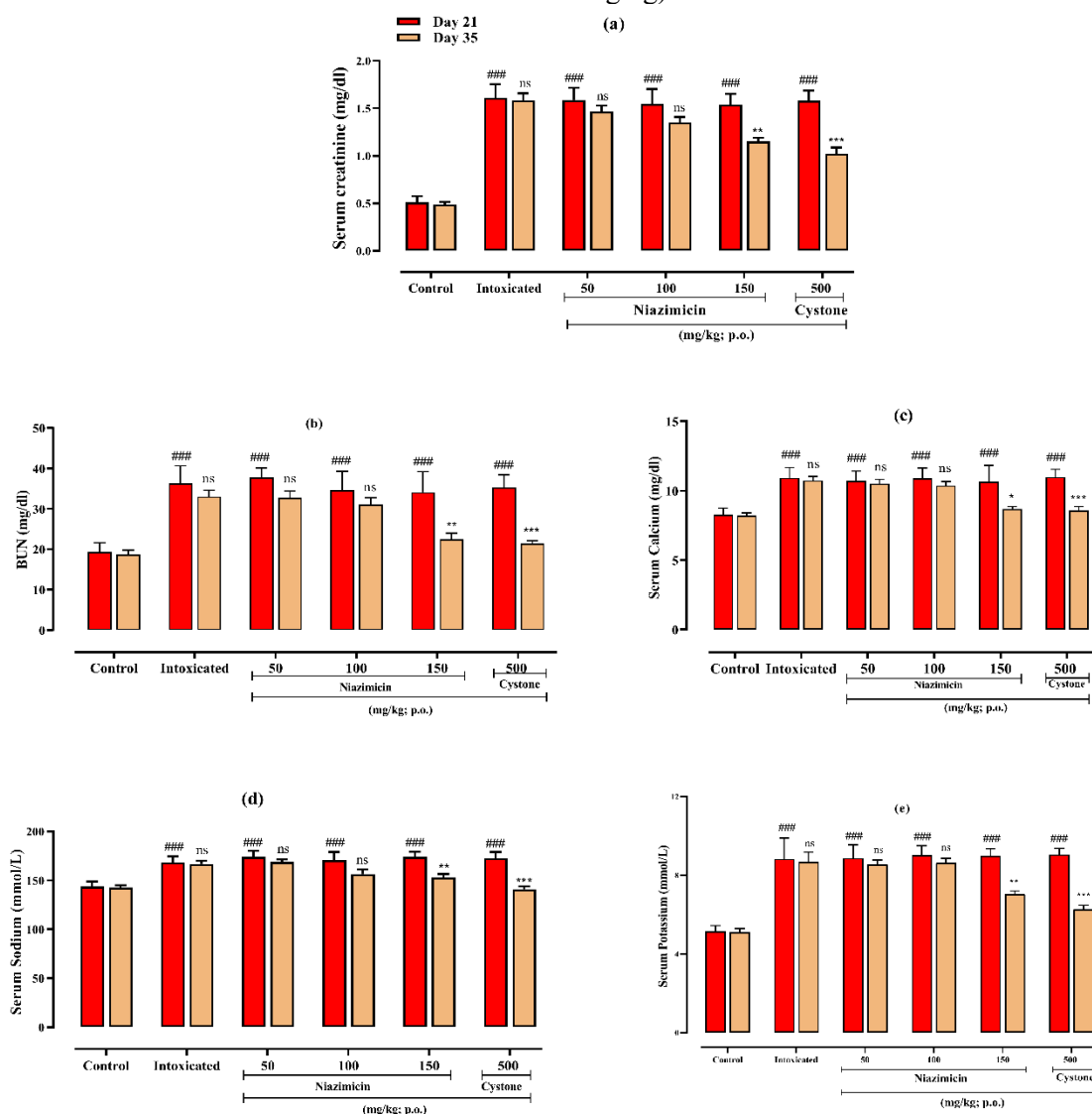


Figure-11. The effects of NZ and Cystone on serum parameters (Mean±SEM; n = 4), two-way ANOVA followed by Bonferroni's post hoc test. Level of significance: ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs intoxication after 21 days and ###: $p < 0.001$ vs control group.

Since ethylene glycol-induced hyperoxaluria most closely mimics the pathogenesis of kidney stone formation in humans, it is considered to be the most valuable animal model for preclinical studies on therapeutic herbs and plants (Atmani et al., 2004). EG raises the level of oxalate in the urine. Elevated oxalate levels in urine bind with calcium to form a compound that leads to crystallization. NZ significantly ($p < 0.001$) reduced the crystal count, which may be due to its spasmolytic potential. This potential can be linked to medical expulsive therapy (MET), which is used to treat urolithiasis (Gilani et al., 1994). Inebriated rats excreted more urine than the normal control group, presumably due to disruption of the renal epithelial membrane, which inhibited water reabsorption. NZ treatment reduced polyuria induced by the lithogenic treatment. Urine output remained higher than that of the normal control group, however, which is in accord with these compounds' intrinsic diuretic action as demonstrated in this study and previously reported (Cáceres et al., 1992; El-Tantawy et al., 2024).

The urolithic group had lower urinary pH, likely as a result of the acidifying influence of ammonium chloride and ethylene glycol to form calculi. An acid pH will promote the crystallization of calcium oxalate (CaOx) crystals, whereas an alkaline pH supports calcium phosphate (CaP) stones (Kamel et al., 2007). In this investigation, NZ showed neutralizing effects, probably due to its urine-alkalinizing potential.

Lithogenic rats had elevated uric acid and magnesium deficiency urinary levels, raised phosphorus excretion, and proteinuria, and all these findings played a key role in oxalate calcium crystal formation and aggregation. The elevation of uric acid blocks glycosaminoglycans and coexists with the calcium oxalate crystals, while magnesium deficiency slows down the availability of soluble complexes of oxalate, creating supersaturation and crystallization. Increased phosphorus excretion stimulates calcium phosphate crystal formation, raising calcium oxalate deposition and inflammation. Proteinuria reflects renal tubular failure and is a nidus for crystal formation. NZ treatment significantly diminished these risk factors through the reduction of uric acid, restoration of magnesium, reduction of phosphorus excretion, reduction of proteinuria, and reduction of the risk of urolithiasis and crystal formation (Al-Wahaibi et al., 2024).

Similar to the Mosquera's work (Mosquera et al., 2020), current study showed increased levels of serum creatinine, BUN, calcium, sodium and potassium.

Kidney stones decrease the efficiency of glomerulus filtrate and also reduce urine filtration leading to the accumulation of nitrogenous substances such as creatinine and BUN in blood. An electrolyte imbalance (Ca^+ , Na^+ and K^+) may disrupt renal cells, which results in structural disorganization of kidney architecture. Thus, maintaining electrolytic equilibrium may lead to the maintenance of kidney cells. Various doses of NZ significantly restored these parameters toward normal. Thus, the curative efficacy of Niazimicin against urolithiasis might be mediated through various pathways such as anticrystallization property, antioxidant effects, spasmolytic potential, upregulation of stone inhibitors, and downregulation of stone promoters. The antiurolithiatic effect of niazimicin may be partially attributed to its reported antioxidant and spasmolytic properties, and these mechanisms should be considered plausible hypotheses requiring further investigation (Gilani et al., 1994). All the animals were monitored daily during the course of the experiment to check their health status. There were no behavioral changes, distress, death or any other visible side effects in any of the treatment groups, including the one given niazimicin, indicating that the tested concentrations were safe for the experimental conditions.

The limitation of this current study is the lack of kidney tissues' histopathological analysis, which would have offered proof of crystal formation in the kidneys as well as tissue damage or protection. Thus, the antiurolithiatic activity noted can only be analyzed within the biochemical and urinary parameters and *in vitro* data generated from this particular experiment. Further studies should consider conducting a thorough histopathology of the kidney tissues, along with other molecular studies, in order to validate any potential tissue protective effect of the studied compounds.

Conclusion

The present study provides preliminary evidence that thiocarbamate glycosides, Niazimicin and Wajidinol, isolated from *Moringa oleifera* seeds, can modulate key processes involved in calcium oxalate crystallization. Niazimicin and Wajidinol demonstrated inhibitory effects on calcium oxalate nucleation, aggregation, and crystal growth through *in vitro* and Niazimicin was associated with reduced urinary crystalluria and improvement in selected biochemical alterations in an experimental model of nephrolithiasis through *in vivo* tests. These findings

suggest that Niazimicin may represent a promising natural product for further investigation in calcium oxalate stone disease. Further adequately powered preclinical studies incorporating renal tissue assessment, pharmacokinetic and safety profiling, and mechanistic investigations are warranted to determine the potential clinical relevance of Niazimicin in the prevention and management of calcium oxalate nephrolithiasis.

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Disclaimer: None.

Conflict of Interest: None.

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Ethical Approval Statement

The study protocols involving animals were approved by the Institutional Review Board (or Pharmacy Animal Ethics Committee) of IUB (No. PAEC/24/109). All animal procedures were conducted in accordance with the relevant guidelines and regulations of IUB/PAEC/24/109 and national standards. The animal sample collection and methods used in this study were conducted in accordance with the ARRIVE guidelines.

Data Availability Statement

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Contribution of Authors

Anjum S: Conceived idea, designed the experiment and supervised the study.

Raza W, Ali H & Jabeen Q: Performed the experiments, collected & analyzed the data and drafted the manuscript.

Shah SAA, Ihsan MZ & Mohiuddin M: Literature review, analyzed data and manuscript editing.

All authors interpreted the data, critically revised the manuscript for important intellectual contents, read and approved the final draft.

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