Isoeugenol Attenuates Oxalate-Induced Renal Injury and Inhibits Glycolate Oxidase: A Natural Strategy Against Nephrolithiasis

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ABSTRACT
Isoeugenol (2-methoxy-4-propenyl-phenol), a natural derivative of eugenol found in plants, essential oils, coffee, and wine, known for its antioxidant, anti-inflammatory, and enzyme-inhibitory properties. With the increasing prevalence of calcium oxalate kidney stones—driven by hyperoxaluria, poor dietary habits, and dehydration—novel, effective treatments are urgently needed. Using NRK-52E renal tubular epithelial cells, we investigated the cytoprotective effects of isoeugenol against oxalate-induced injury. Isoeugenol exhibited no cytotoxicity at concentrations up to 100 µM and significantly improved cell viability in a dose-dependent manner, with viability increasing from 43.79% in oxalate-treated cells to 80.29% at 100 µM. The compound also markedly reduced lactate dehydrogenase release and nitric oxide levels, enhancing membrane integrity and reducing oxidative stress.
Additionally, molecular docking studies demonstrated a strong binding affinity between isoeugenol and glycolate oxidase, implicating a potential mechanism for lowering oxalate synthesis. Collectively, these findings position isoeugenol as a promising, natural, and cost- effective candidate for preventing and managing calcium oxalate urolithiasis, and they underscore the need for further research into its therapeutic applications.

1. Introduction

Isoeugenol, a derivative of eugenol, is naturally present in a variety of sources such as plants, essential oils, coffee, wine, and soaps, and is widely used for flavoring and preservation (Cullere et al., 2004; Bergonzelli et al., 2003). Its conjugated double bond not only contributes to its chemical stability but also enables it to inhibit the formation of superoxide anions mediated by xanthine oxidase (Rajakumar et al., 1993). Recent studies have shown that isoeugenol does not exhibit genotoxicity and appears to have minimal carcinogenic risk under normal exposure conditions; however, these findings underscore the need to consider individual susceptibility and call for further research to fully understand its safety profile (Brock et al., 2024). Furthermore, comprehensive research employing in-silico, in-vitro, and in-vivo approaches has highlighted isoeugenol's neuroprotective potential. Computational analyses suggest that it interacts effectively with targets associated with neurodegenerative diseases, such as acetylcholinesterase and beta-amyloid, while experimental studies have demonstrated its strong antioxidant and anti-inflammatory effects, ultimately protecting neuronal cells from oxidative and inflammatory damage (Alyami et al., 2025).

Parallel to these findings, kidney stones have emerged as a significant and growing health concern worldwide. Their incidence is rising, particularly in developed countries, due to factors such as dietary changes, obesity, and dehydration (Stamatelou & Goldfarb, 2023). Obesity, for instance, is linked to metabolic shifts that increase the urinary excretion of calcium, oxalate, and uric acid, thereby elevating the risk of stone formation (Siener et al., 2004). Additionally, low fluid intake and high sodium diets further predispose individuals to kidney stone development (Cheungpasitporn et al., 2015). While calcium oxalate stones are the most common globally, other stone types like uric acid and struvite are more prevalent in certain regions (Stamatelou & Goldfarb, 2023). Environmental factors, particularly high temperatures, also correlate with an increased incidence of kidney stones (Romero et al., 2010), and genetic predispositions further complicate the risk profile (Tsimihodimos et al., 2016). Consequently, effective management of kidney stone disease requires a multifaceted approach, including lifestyle modifications and public health interventions.

A critical enzyme in oxalate production is glycolate oxidase, which is located in the liver and catalyzes

the conversion of glycolate into glyoxylate—a precursor in oxalate synthesis (Shirfule et al., 2011). In conditions such as primary hyperoxaluria type 1, inhibiting glycolate oxidase has shown promise in reducing excessive oxalate production (Zabaleta et al., 2018). Given the increasing burden of kidney stone disease and the need for safer, more effective treatments, this paper investigates the potential of isoeugenol as an anti-urolithiatic agent. By evaluating its protective effects against oxalate-induced injury in renal tubular epithelial cells, as well as its antioxidant, anti-inflammatory, and glycolate oxidase inhibitory properties, our study aims to provide comprehensive insights into the therapeutic promise of isoeugenol in preventing calcium oxalate stone formation.

2. Materials and methods

2.1. Cell Culture

NRK-52E cells, originating from normal rat renal tubular epithelium, were acquired from the National Centre of Cell Sciences in Pune, India. These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Once the cells reached confluency, they were detached using trypsin and then seeded into 96-well plates at a density of 1×10^4 cells per well. The plates were incubated for 24 hours to allow the cells to adhere properly.

2.2. Evaluation of Isoeugenol Cytotoxicity

The MTT assay was employed to assess the viability of NRK-52E cells following isoeugenol exposure. Cells were plated in 96-well plates at concentrations ranging from 1×10^4 to 1×10^5 cells per well and incubated for 24 hours at 37°C in an atmosphere containing 5% CO_2 and 95% air. After this initial incubation, the cells were exposed to concentrations of 20–100 µM isoeugenol for an additional 24 hours. Subsequently, a solution of MTT (5 mg/mL) was added to each well, and the cells were incubated for 4 more hours to facilitate the formation of formazan crystals. These crystals were dissolved by adding 100 µL of DMSO and mixing thoroughly. Absorbance was then measured at 570 nm using a microplate reader, and the viability of the cells was calculated as a percentage relative to untreated controls. This experiment was repeated at least three times, with each condition tested in triplicate (Mosmann, 1983).

2.3. Assessment of Isoeugenol's Repair Effect on Oxalate-Induced Injury

For this experiment, cells were seeded in 96-well plates at a density of 1×10^5 cells/mL and allowed to adhere for 24 hours in DMEM. The cells were then allocated into four experimental groups:

- Background Control: Wells containing only cell-free medium
- Normal Control: Wells with serum-free medium
- Damaged Group: Cells exposed to 2.0 mmol/L sodium oxalate for 3.5 hours
- Repair Group: Cells treated with 20–100 µM of the test compound for 24 hours

After treatment, the optical density (OD) was measured at 540 nm to evaluate the extent of cell repair, thereby assessing the potential of the phytochemical to reverse sodium oxalate- induced damage (Han et al., 2019).

2.4. Lactate Dehydrogenase (LDH) Assay

Following the treatment period, the culture medium from each well was collected. A reaction mixture, composed of TRIS buffer, lithium lactate, and a PMS-INT-NAD solution, was prepared and dispensed into a 96-well plate. The PMS-INT-NAD solution was formulated by combining INT, PMS, and NAD solutions, and it was kept protected from light. Then, 50 µL of the collected medium was added to each well, and the plate was incubated at 37°C for 5 minutes. Absorbance readings were taken at 490 nm at both 0 and 2 minutes, and the change in absorbance per minute was used to calculate the LDH activity (U/I).

2.5. Determination of Nitric Oxide Levels in the Supernatant

Cells were cultured in 96-well plates at a density of 1×10^6 cells/mL in DMEM. Isoeugenol was introduced as a potential nitric oxide (NO) inducer, while sodium oxalate was used to simulate crystallization. After 24 hours, the levels of NO in the cell supernatant were determined using the Griess reagent method, with absorbance measured at 550 nm. Nitrite concentrations were calculated by reference to a sodium nitrite standard curve. This assay was performed three times, with all samples analyzed in triplicate to ensure accuracy (Kazana et al., 2023).

2.6. Molecular Docking Studies

Molecular docking was conducted using AutoDock Vina to explore the binding interaction between isoeugenol and the target enzyme glycolate oxidase (PDB ID: 2RDT). The protein was prepared in the pdbqt format. Isoeugenol was obtained in sdf format from Drug Bank, processed, and then converted into pdbqt format. The docking simulations were run, and the resulting complexes were visualized to assess binding interactions (Trott and Olson, 2010; Knox et al., 2024).

2.7. Statistical Analysis

All experimental data were collected in triplicate and analyzed using one-way ANOVA. Comparisons among different groups were carried out using Tukey's multiple comparisons test.

3. Results

3.1. Cytoprotective Effects of Isoeugenol Against Oxalate-Induced Injury

Isoeugenol was tested for its ability to protect NRK-52E cells from oxalate-induced damage using an MTT assay at concentrations ranging from 20 to 100 µM. No cytotoxic effects were observed at any of these concentrations—even at 100 µM—confirming the compound's safety for cell-based studies (Figure 1). Because the IC_{50} value was determined to be higher than 100 µM (data not shown), the chosen dose range of 20–100 µM was deemed appropriate for further investigation.

When cells were exposed to sodium oxalate, cell viability dropped significantly to 43.79 ± 1.05%. In contrast, co-treatment with isoeugenol resulted in a concentration- dependent recovery of cell viability. Specifically, viability increased to 57.4 ± 1.7% at 20 µM, 65.93 ± 1.3% at 40 µM, 71.28 ± 0.3% at 60 µM, 74.33 ± 1.49% at 80 µM, and reached 80.29 ± 0.91% at 100 µM. These results clearly indicate that isoeugenol provides significant cyto-protection against oxalate-induced injury.

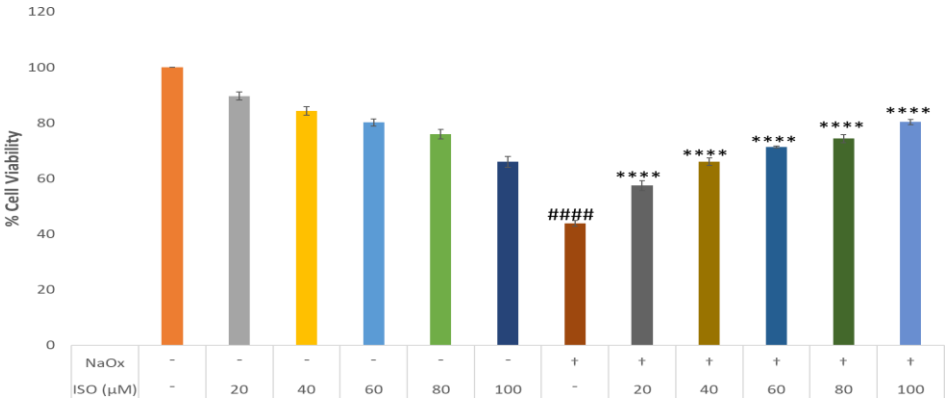

Figure 1: Effect of Isoeugenol (ISO) at different concentrations in cell culture using NRK- 52E on inhibition of percent cell viability. Values are expressed as mean ± SEM (n = 3). Data analyzed with one way ANOVA and Tukey's multiple comparisons test. ####P< 0.0001 vs control, ****P < 0.0001 vs diseased group.

3.2 Lactate Dehydrogenase (LDH)

LDH is a cytoplasmic enzyme that leaks into the surrounding medium when the cell membrane is compromised (Jin et al., 2015). To evaluate the extent of cell damage, LDH activity was measured in the culture medium. As shown in Figure 2, oxalate treatment markedly increased LDH levels—from 0.96 ± 0.08 µM in the control group to 2.38 ± 0.04 µM in the oxalate-treated cells—indicating significant membrane disruption. However, when cells were simultaneously treated with isoeugenol, there was a notable reduction in LDH release. This suggests that isoeugenol helps to maintain cell membrane integrity and reduce overall cellular injury.

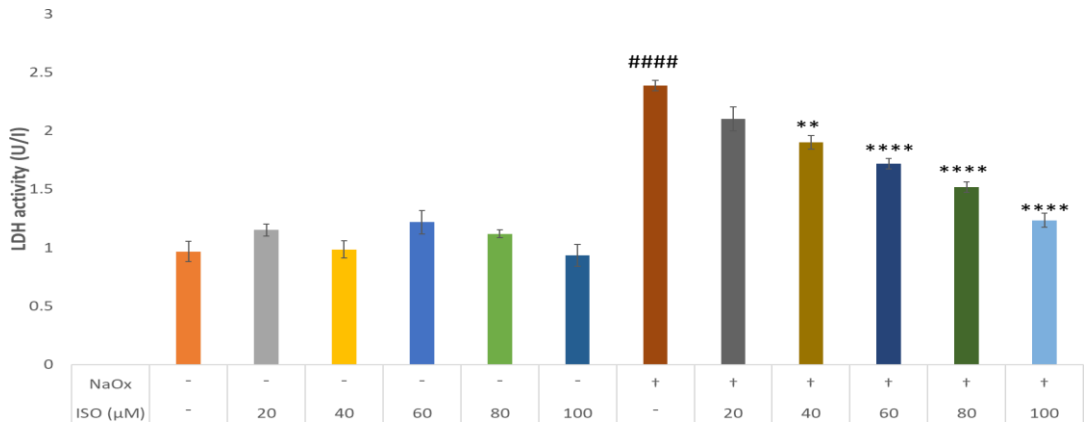

Figure 2: Effect of Isoeugenol (ISO) at different concentrations in cell culture using NRK- 52E on LDH activity. Values are expressed as mean ± SEM (n = 3). Data analyzed with one way ANOVA and Tukey's multiple comparisons test. ####P< 0.0001 vs control, **P< 0.01, ****P < 0.0001 vs diseased group

3.3 **Nitric Oxide Scavenging Assay**

Nitric oxide is a critical mediator of oxidative stress, and its levels were indirectly assessed by measuring nitrite using the Griess reagent. Figure 3 demonstrates that exposure to oxalate resulted in an approximately threefold increase in nitrite levels compared to control cells, indicative of heightened oxidative stress. In contrast, pre-treatment with isoeugenol significantly lowered nitrite concentrations (****P < 0.001), suggesting that isoeugenol effectively mitigates NO production and thus the oxidative damage associated with oxalate exposure.

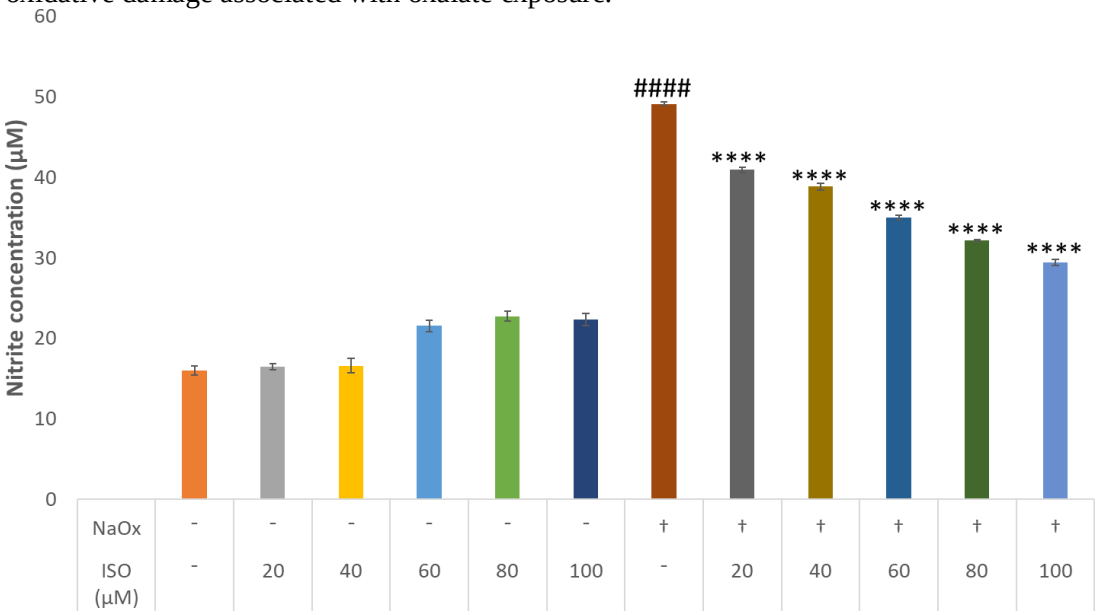

Figure 3. Effect of Isoeugenol (ISO) at different concentrations in cell culture using NRK- 52E on inhibition of nitric oxide concentration in the cell supernatant and (B) percent cell viability. Values are expressed as mean ± SEM (n = 3). Data analyzed with one way ANOVA and Tukey's multiple comparisons test. ####P< 0.0001 vs control, ****P < 0.0001 vs diseased group

3.4 **Molecular docking**

Molecular docking studies were conducted using AutoDock Vina to explore the interaction between isoeugenol and glycolate oxidase (PDB ID: 2RDT). Isoeugenol exhibited a binding affinity of –5.5 kcal/mol, which is comparable to the –5.8 kcal/mol observed for the co- crystallized ligand. Key interactions were identified with residues TYR208, MET82, VAL209, TRP110, LEU164, LEU205, and TYR26 (Figure 4). These docking results suggest that isoeugenol may interact directly with glycolate oxidase, potentially contributing to its protective effects against oxalate-induced injury.

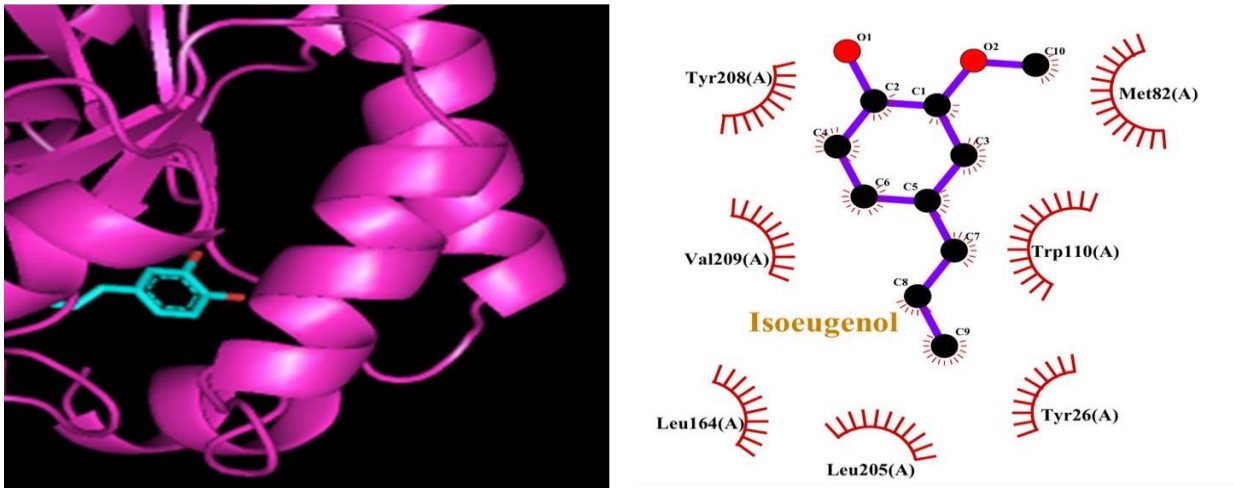

Figure 4: Docking of Isoeugenol and glycolate oxidase in 3D (left) and 2D (right).

4. Discussion

Although significant advances have been made in urology, a fully effective treatment to dissolve kidney stones or prevent their recurrence remains elusive. This gap in therapy often drives clinicians to explore complementary medical systems (Ghelani et al., 2016). One of the primary risk factors for calcium oxalate (CaOx) stone formation is hyperoxaluria. Elevated oxalate levels can damage renal tubular epithelial cells by altering their structure, compromising membrane integrity, increasing free radical production, and reducing antioxidant defenses, which collectively lead to cell death. Such cellular injury promotes the adhesion and retention of CaOx crystals, a critical step in the development of urolithiasis (Hong et al., 2023; Zuo et al., 2011). In our study, NRK-52E cells exposed to oxalate ions exhibited marked damage, underscoring the role of cell injury in the pathogenesis of kidney stones.

Isoeugenol demonstrated a notable protective effect against this oxalate-induced damage. Its nephroprotective action may be largely attributed to its antioxidant capabilities, particularly its ability to scavenge nitric oxide (NO). The release of lactate dehydrogenase (LDH), a stable cytosolic enzyme used as a marker for cell injury (Aggarwal et al., 2017), was significantly elevated in oxalate-treated cells. However, when isoeugenol was introduced, LDH release was reduced, and cell viability improved, suggesting that isoeugenol helps maintain cell membrane integrity and mitigate injury. Additionally, the natural origin of isoeugenol offers the advantage of being a less expensive and safer alternative to synthetic compounds (Kumar et al., 2011).

Inflammation is another crucial factor in kidney stone formation. Kidney tissues from stone- forming patients often exhibit upregulated inflammatory gene expression. The interaction between renal cells and CaOx crystals can provoke an inflammatory response, leading to the activation of macrophages into a pro-inflammatory M1 phenotype. These cells secrete cytokines such as MCP-1, TNF-α, and IL-6, which in turn boost NO production and contribute to crystal aggregation (Liu et al., 2022; Ahmatjan et al., 2023; Thongboonkerd et al., 2021).

The anti-inflammatory and antioxidant properties of isoeugenol may therefore be key to its ability to protect renal cells from oxalate-induced injury.

5. Conclusion

In summary, isoeugenol effectively safeguards NRK-52E renal tubular epithelial cells against oxalate-induced injury by enhancing cell viability and reducing markers of cellular damage, such as LDH release. This protective effect likely interferes with the processes that facilitate CaOx stone formation and retention. Furthermore, molecular docking studies indicate that isoeugenol can inhibit glycolate oxidase, an enzyme involved in oxalate metabolism, adding to its potential as an anti-urolithiasis agent. Collectively, these findings highlight isoeugenol's promising role as a natural, cost-effective, and safe candidate for preventing and treating calcium oxalate urolithiasis.

6. Acknowledgement

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