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Inhibitory effects of *Moringa oleifera* seed extracts on crude xanthine oxidase

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Abstract

The quest for novel xanthine oxidase (XO) inhibitors, characterized by reduced side effects and heightened therapeutic efficacy, holds significant importance in addressing hyperuricemia and gout. This study was undertaken to assess the inhibitory properties of *Moringa oleifera* seed extracts on crude XO. The isolation of crude XO from bovine (cow) milk was achieved through ammonium sulfate precipitation techniques, and its activity was subsequently quantified using spectrophotometric measurements at a wavelength of 293 nm. The impact on xanthine oxidase inhibition was investigated utilizing varying concentrations of *Moringa oleifera* seed extracts in methanol, ethyl acetate, and aqueous solutions. Among these extracts, the ethyl acetate variant displayed the most robust inhibitory effect, with a notable inhibition rate of 89.16% ± 0.42, followed by the methanol extract at 65.59% ± 0.79 and the aqueous extract at 48.77% ± 0.85. For comparison, the positive control (allopurinol) exhibited an inhibition rate of 94.32% ± 0.66. The kinetic parameters of the enzyme were determined, revealing V_{max} and k_m values of 0.05 U/ml and 131.35 µM, respectively. When the enzyme was exposed to the inhibitory extracts, the resulting V_{max} and k_m values were as follows: ethyl acetate extract; 0.03 U/ml and 77.12 µM, methanol extract; 0.04 U/ml and 131.35 µM, and aqueous extract; 0.02 U/ml and 74.23 µM. This research work establishes a foundation for the potential utilization of *Moringa oleifera* seed extracts in addressing hyperuricemia and, consequently, gout.

Keywords: Xanthine oxidase; *Moringa oleifera*; Inhibition; Hyperuricemia

1. Introduction

Xanthine oxidase (XO), an essential multifunctional metallo-flavoprotein containing molybdenum, exhibits broad distribution across various sources, such as milk, microorganisms (bacteria), and animal organs (heart, lungs, and kidneys) [1]. In the realm of human physiology, XO plays a vital role in purine metabolism [2]. Specifically, within the process of uric acid synthesis, XO facilitates the oxidation of hypoxanthine to xanthine and further converts xanthine into uric acid through enzymatic reactions [3]. This resultant uric acid is subsequently eliminated via urine. The overproduction of uric acid, attributed to hyperuricemia, leads to conditions such as gout [4]. To counter this, XO inhibitors such as allopurinol block the final step of uric acid metabolism and are commonly employed for gout treatment [5]. Hyperuricemia, a condition arising from an imbalance in uric acid excretion, can be induced by excessive consumption of certain dietary components, such as meats, select seafood, and nucleic acid-rich foods. The manifestation of hyperuricemia is characterized by elevated plasma uric acid levels, reaching up to 7.0 mg/dl in males and 5.7 mg/dl in females [6]. This condition is closely associated with a spectrum of disorders, including cardiovascular ailments, renal dysfunction, and gout [7].

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Natural products have gained recognition as valuable reservoirs of phytochemicals harboring unique pharmacological attributes. Within plant extracts, including those from vegetables and herbs, numerous enzyme inhibitors have been pinpointed and effectively isolated [8]. Among these phytochemicals, polyphenols have captured considerable scientific attention, primarily due to their diverse array of biological functions encompassing antioxidative, anti-inflammatory, antimicrobial, anticarcinogenic, and enzyme-inhibitory properties [9]. These polyphenols, derived from plant-based diets, have been harnessed traditionally for medicinal purposes, particularly in the management of hyperuricemia and gout [10]. In the context of polyphenols, with a focus on flavonoids, their capacity to curtail xanthine oxidase activity has come to light, implying their potential as novel agents for crafting xanthine oxidase inhibitors tailored for hyperuricemia and gout therapy [11].

Moringa oleifera, a member of the Moringaceae family, stands out as an immensely valuable botanical entity colloquially referred to as 'Zogale' in the northern regions of Nigeria. Esteemed for its notably robust nutritional profile, this herbal plant plays dual roles in Nigerian culture, finding utility both as a dietary staple and as a medicinal resource [12]. The comprehensive array of phytochemicals—ranging from flavonoids and terpenoids to saponins and alkaloids—pervades diverse segments of the *Moringa oleifera* plant, encompassing its seeds, stem, root, leaves, flowers, and bark [13]. Notably, the plant's anti-inflammatory and antimicrobial attributes have propelled it into the spotlight as a promising contender for therapeutic application [13]. Within the confines of this investigation, the study delves into the inhibitory capacities of methanolic, ethyl acetate, and aqueous extracts sourced from *Moringa oleifera* seeds. The primary focus centers on the impact of these extracts on the activity of isolated xanthine oxidase, an enzyme of interest.

2. Materials and methods

2.1. Sample collection

Fresh cow milk was procured from Maizube Farms, located in Minna, Niger State. *Moringa oleifera* seeds, obtained in June 2021, were sourced from Engr. Kure Market in Minna, Niger State. These seeds were subjected to authentication by the Department of Plant Biology at the Federal University of Technology in Minna, Niger State.

2.2. Preparation of plant samples

The *Moringa oleifera* seeds underwent a dehusking process to reveal the seed kernels. These kernels were then air-dried over three weeks. Following the drying phase, the kernels were finely ground into a smooth powder using a conventional kitchen-style electric blender. The resulting powder was accurately weighed and subsequently placed within an airtight container for safekeeping until subsequent utilization [14-15].

2.3. Isolation of XO from Bovine (Cow) Milk

The method for extracting XO from cow milk was adapted from [16] with certain adjustments. Fresh cow milk was centrifuged using a high-speed refrigerated centrifuge (LR10-24A) at 5000 rpm for 15 minutes, facilitating the separation of the cream fraction. This obtained cream was subsequently mixed with a 250 ml solution of 0.2 M sodium phosphate buffer (pH 7.3) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and stirred for 90 minutes. Next, the mixture was subjected to centrifugation at 2500 rpm for 30 minutes, leading to the removal of the supernatant. To the remaining residue, 37 ml of cold butanol (15%) was cautiously added, followed by a gradual addition of ammonium sulfate (15%) while continuously stirring for 1 hour. After the stirring process, the entire mixture underwent further centrifugation at 2500 rpm for 20 minutes. The resulting supernatant was discarded, and the remaining residue was subjected to an additional step wherein ammonium sulfate (20%) was gradually introduced while stirring continuously for 30 minutes. The resulting mixture was left to stand overnight. After overnight incubation, the residue was once again subjected to centrifugation at 2500 rpm for 30 minutes. The resultant white precipitate, serving as the crude enzyme, was collected, and its weight was determined (28.37 g) [14, 16].

2.4. Determination of Crude XO Activity

The method for assessing the activity of the isolated XO in its crude form was adapted from [17] while incorporating certain adjustments. Initially, a test tube was employed, into which 1.9 ml of monobasic sodium phosphate buffer (pH 7.3) was pipetted. Subsequently, 1 ml of a 0.15 mM xanthine solution was introduced into the test tube, and the contents were thoroughly mixed. To this mixture, 0.1 ml of the crude isolated enzyme solution was promptly added and mixed by inversion. The absorbance of this composite solution was immediately measured at a wavelength of 293 nm using a UV–visible spectrophotometer. Following the initial reading, a subsequent absorbance measurement was taken after a 10-minute interval. This was executed to ascertain the augmentation in absorbance stemming from the release of uric

acid during the enzymatic reaction. Sequential absorbance measurements were then conducted at 10-minute intervals for over one hour. As a reference, distilled water was employed as the blank in these measurements [17].

2.5. Determination of the Effect of Substrate Concentration on the XO-Catalyzed Reaction

A test tube was utilized for this process, into which 1.9 ml of sodium phosphate buffer with a pH of 7.3 was carefully pipetted. Subsequently, 1 ml of a 0.10 mM xanthine solution was introduced into the test tube and thoroughly mixed. Next, 0.1 ml of crude XO was added to the mixture and gently mixed by inversion. The resultant increase in absorbance was then recorded at a wavelength of 293 nm using a UV–visible spectrophotometer. This entire procedure was replicated using xanthine solutions of four distinct concentrations (0.15, 0.20, 0.25, and 0.30 mM) to encompass a range of concentrations [18].

2.6. Extraction of *Moringa oleifera* seeds

Adhering to certain adaptations, the process of extracting *Moringa oleifera* seeds was executed following the outline presented in [19]. Initially, the finely ground seed kernels were partitioned into three equal portions, each amounting to 100 grams. Subsequently, in separate beakers, 300 ml of distilled water, methanol, and ethyl acetate were individually added to the portions. These mixtures were subjected to maceration for 72 hours. Following the maceration period, the mixtures underwent filtration using a fine mesh cloth, and the resultant extracts were then concentrated using a water bath set at a temperature of 23 °C. The extracts obtained from this process were meticulously collected and subsequently stored in a refrigerator for future utilization. Notably, the ethyl acetate extract yielded a distinctly oily substance, while the methanol and aqueous extracts yielded brown, adhesive semisolid materials [19].

2.7. Determination of the Inhibitory Activity of Ethyl Acetate, Methanol, and Aqueous Extracts on XO Activity

Using a 50 mM solution of monobasic sodium phosphate buffer (pH 7.3) as the medium, distinct concentrations (1, 2, and 3 mg/ml) of ethyl acetate, methanol, aqueous extracts, and allopurinol were dissolved. Each solution was then introduced into separate test tubes, each containing 0.9 ml of monobasic sodium phosphate buffer (pH 7.3) and 1 ml of enzyme solution. Thorough mixing was ensured, followed by an incubation period at room temperature lasting for 30 minutes. Post incubation, an additional 1 ml of a 0.15 mM xanthine solution was added to each test tube to initiate the reaction. After thorough mixing, a subsequent incubation of 20 minutes was carried out. The absorbance of the resulting mixture was recorded at 293 nm utilizing a UV–visible spectrophotometer, with distilled water serving as the blank reference [17]. To determine the percentage inhibition of XO activity for each *Moringa oleifera* extract, the following formula was employed:

$$Y = \frac{X - X_1}{X} \times 100$$

where Y = percent inhibition, X= XO activity (without extract), and X_1 = XO activity (with extract).

3. Results

3.1. Determination of Crude XO Activity

Figure 1, depicted below, illustrates the impact of reaction time on the activity of the crude XO. Notably, an observable trend emerged wherein the XO activity demonstrated an upward trajectory alongside an extension in the reaction time.

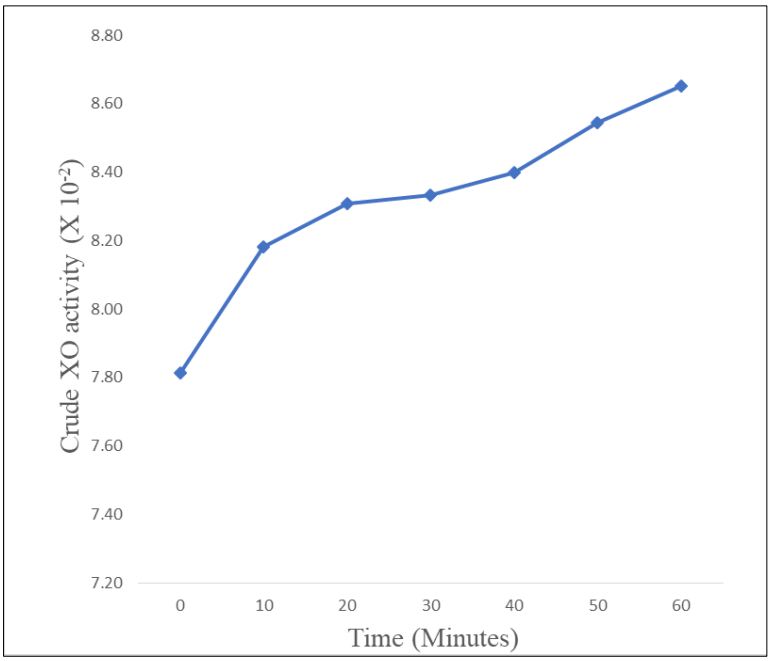

Figure 1 Effect of Reaction Time on Activity of Crude XO

3.2. Percentage Inhibition of Crude XO by Methanolic, Ethyl Acetate, and Aqueous Extracts

Figure 2 shows the recorded percentages of inhibition about the crudely isolated XO, as influenced by varying concentrations of *Moringa oleifera* seed extracts and the positive control (allopurinol). Notably, a marked distinction emerged when the methanol extract was compared to allopurinol, showcasing a notably higher percentage of inhibition ($p < 0.05$). In contrast, the ethyl acetate and aqueous extracts demonstrated comparatively lower percentages of inhibition.

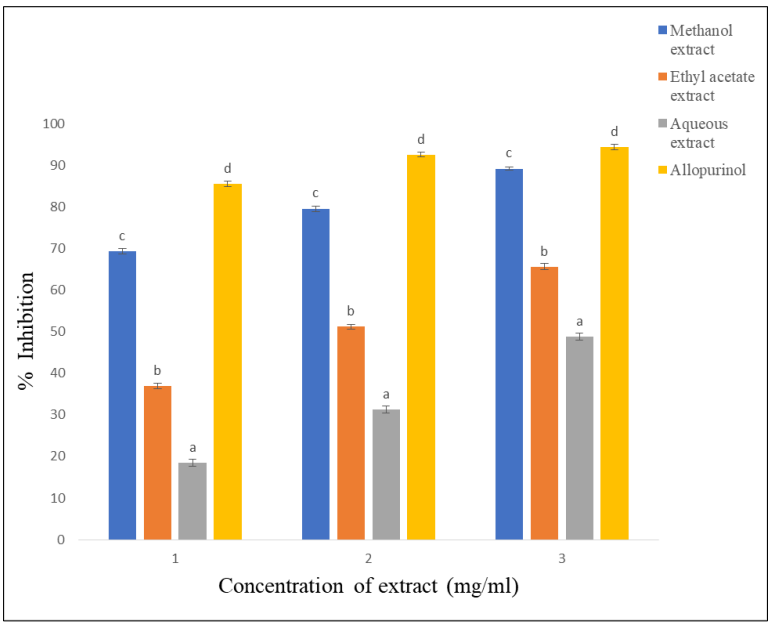

Figure 2 Inhibitory Effects of Extracts on **Crude** XO Activity

3.3. Effect of Substrate and Enzyme Concentration on the Activity of Crude XO

Displayed in Figure 3 is a Michaelis–Menten plot illustrating the influence of substrate concentration (xanthine) in mM on the activity of the crude XO.

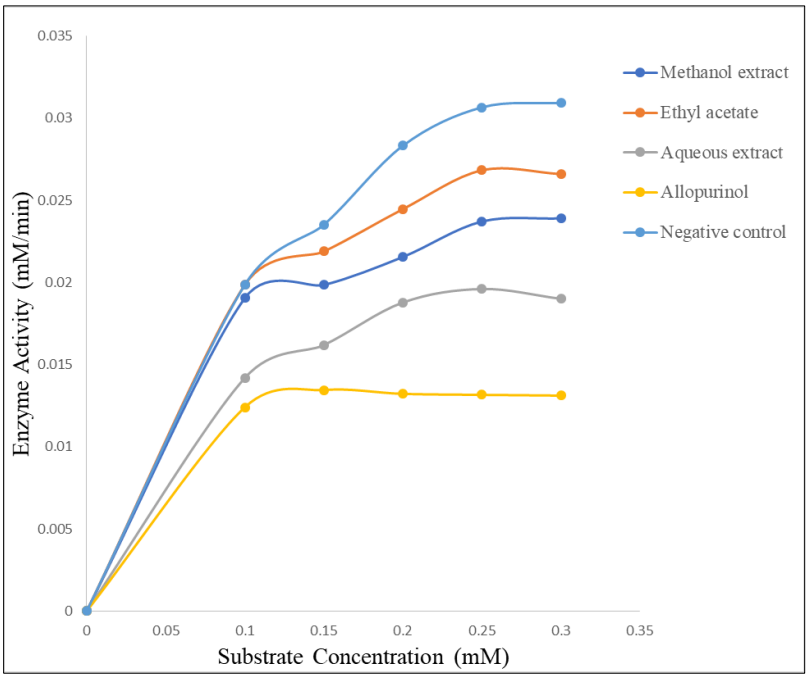

Figure 3 Effect of Substrate and Extract Concentrations on Enzyme Activity

3.4. Line Weaver-Burk Plot of Inhibition of XO Activity by Methanol Extract

As depicted in Figure 4 below, the methanol extract induced a decrease in the maximum velocity (V_{max}) of the XO yet left the Michaelis constant (K_m) unaffected. Specifically, the methanol extract led to a reduction in XO's Vmax from 0.05 U/mL to 0.04 U/mL, while the K_m remained constant at 131.35 µM.

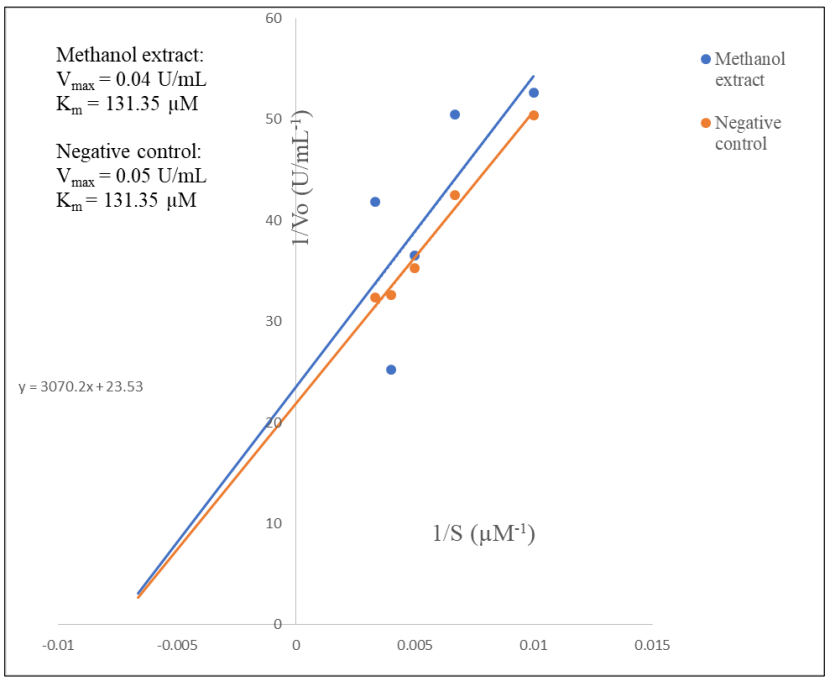

Figure 4 Inhibition of **Crude** XO by Methanol Extract

3.5. Line Weaver-Burk Plot of Inhibition of XO Activity by Ethyl Acetate Extract

As illustrated in Figure 5 below, the introduction of the ethyl acetate extract led to a decline in both V_{max} and K_m, mirroring the impact akin to that observed with allopurinol. Specifically, the ethyl acetate extracts reduced V_{max} and K_m,

shifting them from their original values of 0.05 U/mL and 131.35 µM to new levels of 0.03 U/mL and 77.12 µM, respectively.

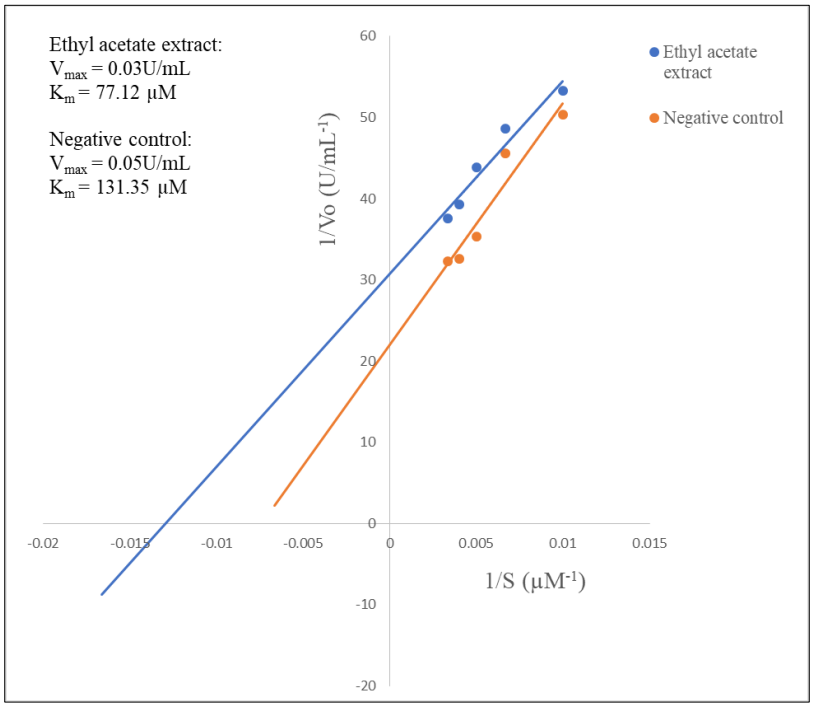

Figure 5 Inhibition of **Crude** XO by ethyl acetate extract

3.6. Line Weaver-Burk Plot of Inhibition of XO Activity by the Aqueous Extract

As presented in Figure 6, the aqueous extract, akin to the ethyl acetate extract and allopurinol, triggered a reduction in both V_{max} and K_m for XO. Notably, the aqueous extract elicited decreases in the V_{max} and K_m of XO, leading to new values of 0.02 U/mL and 74.23 µM, respectively.

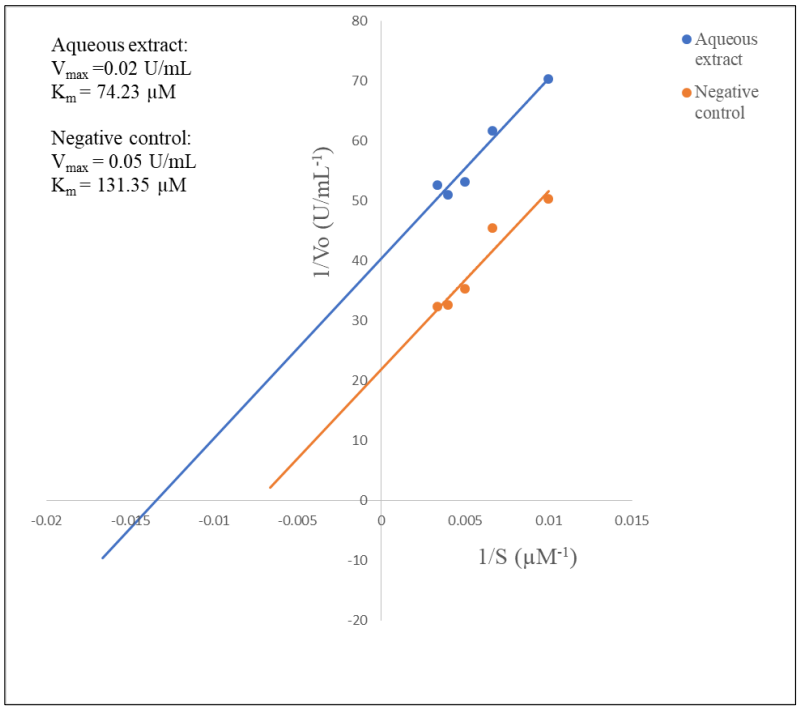

Figure 6 Inhibition of Crude XO by Aqueous Extract

4. Discussions

Traditionally, plants and herbs have held a significant role in the management of hyperuricemia and gout. However, in contemporary times, the exploration of potential XO inhibitors sourced from plants has emerged as a natural avenue for the development of prospective treatments for hyperuricemia [20]. Within *Moringa oleifera* seeds lie potent polyphenols known for their XO inhibitory properties. This study delves into the invitro investigation of the inhibitory potential of methanol, ethyl acetate, and aqueous extracts from *Moringa oleifera* seeds on crude XO activity. This inquiry is carried out through the quantification of uric acid absorbance generated during the XO-catalyzed reaction. This endeavor aligns with the methodologies employed by [15], where XO activity was also assessed spectrophotometrically [15].

Isolated from cow milk, crude XO underwent extraction, and its catalytic activity was gauged through the quantification of uric acid generated at 10-minute intervals, as demonstrated in Figure 1. Notably, a publication by [21] indicated that the temporal progression of XO activity in a catalyzed reaction exhibited an incremental trend, showcasing an elevation in enzyme activity over time [21]. The inhibitory impact of the extracts on XO was observed to be concentration-dependent, a phenomenon exemplified by escalating inhibition percentages corresponding to increasing extract concentrations (Figure 2). In juxtaposition with the potent inhibition exerted by allopurinol (94.32%) at a concentration of 3 mg/mL, the ethyl acetate extract demonstrated the highest inhibition percentages (89.6%) against crude XO. This was trailed by the methanol extract (65.59%) and, finally, the aqueous extract (48.77%). Remarkably, these outcomes harmonize with the percentage inhibition results derived from the ethanolic extract of *Moringa oleifera* seeds as outlined in the study conducted by [22].

The influence of the substrate (xanthine) concentration on the catalyzed reaction of crude XO was also examined. Under the negative control conditions (absence of an inhibitor), the reaction rate, denoting enzyme activity, displayed a proportional relationship with the substrate concentration. In other words, the activity of the crude XO escalated until it reached its maximum velocity, a point at which the enzyme's active sites became thoroughly saturated with the substrate. This outcome aligns seamlessly with the findings presented in [17]. However, with the introduction of *Moringa oleifera* seed extracts at a concentration of 3 mg/mL, a reduction in enzyme activity was observed in contrast to the uninhibited enzyme state (as depicted in Figure 3). This resemblance to the inhibitory effects identified in [23] further corroborates the inhibitory behavior. The underlying mechanism for this inhibitory effect can be attributed to the polyphenolic composition within *Moringa oleifera* seed extracts, specifically the presence of flavonoids. Notably, flavonoids are recognized as potent XO inhibitors [24]. A parallel perspective was highlighted by [25], wherein it was emphasized that flavones contribute significantly to the inhibition of XO activity, provided they remain unaltered during the extraction process [25].

Conducting kinetic investigations to discern the inhibition mode exhibited by *Moringa oleifera* seed extracts on crude XO, crucial enzyme kinetic constants were unveiled: a V_{max} (maximum velocity) of 0.05 U/mL and a K_m (Michaelis–Menten constant) of 131.35 µM. Remarkably, these values mirrored those documented in [24]. Similar findings were observed in the works of Chioma *et al.* and Sakti *et al*., who reported congruent values of 0.05 U/mL and 131.35 µM for K_m and V_{max}, respectively, under extract-absent conditions [26, 15]. Notably, the V_{max} activity remained unaffected [26, 15]. Intriguingly, the Line-Weaver Burk plot portraying the ethyl acetate extract of *Moringa oleifera* seeds showed an uncompetitive inhibition pattern (depicted in Figure 5), akin to the aqueous extract (as shown in Figure 6). This mode of inhibition entails XO-inhibiting molecules within the extracts binding exclusively to the enzyme-substrate complex, subsequently leading to a reduction in the enzyme's V_{max}. Simultaneously, the Km is diminished, which is attributed to an enhanced binding affinity between the substrate and the crude XO. Conversely, the Line-Weaver Burk plot for the methanol extract presented a noncompetitive inhibition profile (as portrayed in Figure 4), in alignment with insights from [27]. This highlights the intriguing reality that extracts from the same plant may engender distinct impacts on the enzyme. Noncompetitive inhibition signifies that inhibitor molecules associate with the enzyme post substrate binding, resulting in lowered enzyme activity and V_{max} while maintaining an unchanged K_m due to the inhibitor's lack of influence on substrate affinity for the enzyme [27].

5. Conclusion

In contrast with allopurinol, *Moringa oleifera* seed extracts displayed promising and moderate inhibitory effects on crude XO. Notably, the methanolic extract demonstrated a noncompetitive inhibition pattern, whereas the ethyl acetate and aqueous extracts exhibited an uncompetitive mode of inhibition. This diverse spectrum of inhibition mechanisms underscores the complex interactions between *Moringa oleifera* extracts and the XO enzyme. From a therapeutic vantage point, the observed inhibitory effects of *Moringa oleifera* hold potential significance in treating hyperuricemia

and gout. These effects are attributed to polyphenolic phytochemicals found within *Moringa oleifera* seed extracts. The plant's potential to offer more cost-effective and efficacious alternatives for managing inflammatory-related conditions, including hyperuricemia and gout, emerges as an encouraging prospect.

Recommendation

While the exact inhibitory mechanism of polyphenols found in *Moringa oleifera* on XO remains somewhat elusive, the potential utilization of *Moringa oleifera* seeds for mitigating inflammation associated with hyperuricemia and gout has gained substantial recognition. Nevertheless, it is important to note that a significant portion of these analyses have been conducted *in vitro*, with only a limited number of studies exploring in vivo inhibitory effects. Future research endeavors should emphasize a more comprehensive investigation into the interplay between bioavailability, inhibitory potency, and the potential occurrence of adverse effects. Such exploration will contribute to a deeper understanding of *Moringa oleifera*'s therapeutic potential and aid in its safe and effective application.

Compliance with ethical standards

Acknowledgment

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Disclosure of conflict of interest

No conflict of interest is to be disclosed.

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