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Antilithiasic and antioxidant activities of propolis and bee pollen extracts: *In vitro* and *in silico* studies

Safia Ali Haimoud[1,*], Fatiha Arioui[1], Meriem Medjekane[2], Leila Ali Haimoud[3], Khelifa Bahri[1], Asma Cheikh[1], Saadia Ghiboub[1], Hanane Benabed[1], Ikram Boudali[1], Besma Ayache[1], Chaima Beskri[1]

[1] Hassiba Benbouali University of Chlef, Ouled Fares, Algeria
[2] University Center Nour Bachir, El Bayadh, Algeria
[3] Public Hospital, Chlef, Algeria

* e-mail: s.alihaimoud@univ-chlef.dz

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Abstract:
Urolithiasis is a wide-spread condition with no efficient pharmacological treatments. It causes the formation of renal stones.
The article describes the antioxidant and anti-lithiasis potencies of extracts of Algerian propolis and bee pollen. Their inhibiting effect on calcium oxalate crystallization was assessed against citric acid using UV-Visible spectrophotometry at 620 nm. The effect of rutin and liquiritin on xanthine dehydrogenase was analyzed in silico.
The highest levels ($p < 0.05$) of total phenols (129.28 ± 1.51 mg GAE/g) and flavonoids (77.58 ± 1.95 mg QEQ/g) belonged to the bee pollen ethanolic extract. The same extract had the strongest ($p < 0.05$) DPPH scavenging capacity (9.420 ± 0.002 µg/mL). The ethanolic extract of propolis possessed the highest antioxidant potency (63.05 ± 3.49%) according to the β-carotene assay. Crystal nucleation in the presence of 16 mg/mL extracts was the same as in the samples tested with citric acid. The propolis ethanolic extract (82.83 ± 0.83%) demonstrated the maximal inhibition of crystal growth at 16 mg/mL, compared with citric acid (91.90 ± 0.26%). Based on the binding free energy (ΔG) and root mean square deviation, the ligands were efficient inhibitors of xanthine dehydrogenase.
Bee pollen and propolis proved to contain bioactive components that can make them an alternative to traditional methods of urolithiasis prevention and treatment.

Keywords: Bee products, calcium oxalate, crystallization, extracts, DPPH method, antilithiasic activity, xanthine dehydrogenase

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INTRODUCTION

Urolithiasis is a common disorder that arises from the formation of renal stones and has no efficient pharmacological treatment [1]. Some forms are recurrent; severe cases may lead to kidney disorders, causing pain, bleeding, and urinary tract infections. The stone formation encompasses all processes within the urinary tract and is divided into two primary stages, i.e., nucleation and crystallization. Crystals develop from substances originally dissolved in urine. This stage is not inherently pathological as these crystals can aggregate and grow at various points within the urinary system [1]. Infection appears to facilitate stone formation by inducing physical and chemical changes in urine, including acidification [2]. Urolithiasis depends on health conditions, eating habits, and lifestyle [3]. The most common clinical symptom of this kidney pathology is nephritic colic, which is characterized by severe and sudden pain in the renal region. Urolithiasis affects around 10% of population in non-industrialized regions [4, 5].

Urolithiasis exacerbates the oxidative stress, which is a cytotoxic condition implicated in the development of such pathologies as cancer, cardiovascular disease, hypertension, autoimmune disease, diabetes, multiple sclerosis, and arthritis [6]. Xanthine dehydrogenase facilitates purine metabolism by generating reactive oxygen species, such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2). Xanthine oxidoreductase exists in

two interchangeable forms, i.e., xanthine dehydrogenase and xanthine oxidase. The dehydrogenase form transitions to the oxidase form, which then produces hydrogen peroxide and superoxide. This transformation happens through the oxidation of sulfhydryl groups or via stimulated proteolysis. Both forms of the enzyme can cause the oxidation of nicotinamide adenine dinucleotide hydrogen (NADH) while simultaneously producing reactive oxygen species. Because the xanthine oxidase reaction produces reactive oxygen species and uric acid, this enzyme is considered to be a major source of reactive oxygen species in the bloodstream [7].

Bees produce beeswax, royal jelly, bee pollen, and propolis. The importance of bee pollen comes from its rich complex composition. It includes vitamins (C, E, B-group vitamins), mineral salts (phosphorus, calcium, magnesium, copper, zinc, iron), essential amino acids, and phenolic compounds known for their numerous biological properties [8].

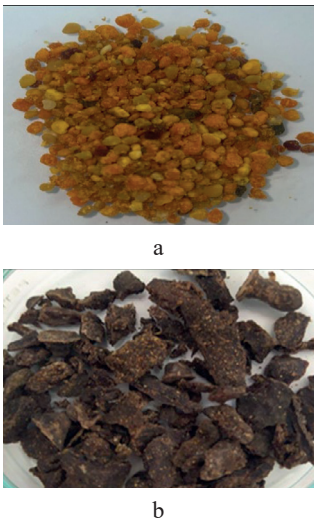
Propolis is a natural resinous substance gathered by bees (*Apis mellifera*). It contains more than 300 constituents, including volatile aromatic compounds, minerals (iron, calcium, zinc, copper, manganese), vitamins (C, E, B-group), aliphatic fatty acids, esters, amino acids, phenolic acids, and flavonoids [9–12].

Bee by-products and their chemical composition receive a lot of scientific attention due to their phenolic components [13]. However, their biological effects remain understudied. For instance, no reliable scientific data are available on the antilithiatic effect of propolis and bee pollen extracts on the crystallization of calcium oxalate monohydrate. This research examined the antioxidant and inhibiting effects of Algerian native propolis and bee pollen on the crystallization of calcium oxalate. The research objective was to unravel possible new applications in medicine as antilithiatic agents, as well as to determine the interaction of rutin and liquiritin with xanthine dehydrogenase *in silico*.

STUDY OBJECTS AND METHODS

Ingredients and chemicals. The chemicals used in this work were of analytical grade. The list of solvents included methanol, ethanol, chloroform, sodium bicarbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, calcium chloride, sodium oxalate, hydrochlorid acid, and butylated hydroxytoluene. They were supplied from Sigma-Aldrich GmbH (Germany). The Folin-Ciocalteu reagent, *β*-carotene, sodium hydroxide, gallic acid, quercetin, linoleic acid, and Tween 40 were obtained from Sigma Chemical (USA). The sodium nitrite and aluminum chloride were supplied by Fluka (Germany).

Beehive by-products. In this research, the by-products of bees were represented by bee pollen and propolis. They were harvested from the Chlef Region, Western Algeria, in February 2020 and 2021, respectively. Bee pollen is bright yellow round grains; propolis is a dark brown substance with a pungent flavor and sweet fragrance. The samples were stored in a freezer at –20°C for further analysis (Fig. 1).



a

b

Figure 1 *Apis mellifera*'s beehive by-products: (a) bee pollen, (b) propolis

Preparing the extracts. To obtain crude methanolic extracts, 2.5 g of propolis and bee pollen were separately added to 25 mL of methanol. The resulting mix was filtered and concentrated using a rotary vacuum evaporator (Büchi, Switzerland) [13]. After that, 10 g of pollen and propolis grain powder were separately added to 100 mL of ethanol. The preparations were stirred magnetically for 30 min. After 24 h, the macerates were filtered and concentrated under vacuum [10].

Bioactive content. ***Total phenolic content.*** To determine the total phenolic content, we shook 200 µL of each extract with 1.5 mL of the Folin-Ciocalteu reagent (1:10) and 1.5 mL of sodium bicarbonate (60 g/L). After 90 min, the absorbance was measured spectrophotometrically at 725 nm using an Optizen 2120 device (Mecasys, South Korea). The total phenolic content was given as 1 mg gallic acid equivalent per 1 g extract (mg GAE/g) [14].

Total flavonoid content. To determine the total flavonoid content, we mixed 1 mL of each extract, 4 mL of distilled water, and 0.3 mL of sodium nitrite (5%). After 5 min, we added 0.3 mL of aluminum chloride (10%), 2 mL sodium hydroxide (4%), and 2.4 mL distilled water. After another 5 min, we measured the optical density at 510 nm. The total flavonoid content was given as 1 mg quercetin equivalent per 1 g extract (mg QEQ/g) [15].

Antioxidant potencies *in vitro. β-Carotene-linoleic assay.* We mixed 1 mL of *β*-carotene (3 mg) dissolved in 30 mL chloroform with 40 mg of linoleic acid and 400 mg of Tween 40. Upon removing chloroform at 40°C, we added 100 mL of distilled water. After that, we added 3 mL of the resulting emulsion to 50 µL of the test sample (20 mg/mL); 10 µg/mL butylated hydroxytoluene served as comparison. The optical density was recorded at 470 nm. The antiradical activity (AA, %) of the test sample was calculated by the following Eq. (1):

$$AA = \frac{A_{t0} - A_{t60}}{A_{t0}} \times 100 \quad (1)$$

where A_{t0} and A_{t60} were the initial absorbance and the absorbance measured after 60 min of incubation for the extracts and the blank, respectively [16].

DPPH radical-scavenging. We incubated the mixes of 2.7 mL extract concentrations of propolis and bee pollen with 0.3 mL of a DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (0.004%) for 30 min. The discoloration was recorded at 517 nm; butylated hydroxytoluene served as comparison. The inhibition activity (I, %) was calculated using the following Eq. (2):

$$I = \frac{A_0 - A_1}{A_0} \times 100 \quad (2)$$

where A_0 and A_1 were the absorbance values of the blank (DPPH solution) and the sample extracts, respectively.

The antiradical potency was expressed in term of IC_{50} (μg/mL), i.e., the scavenging of 50% of DPPH radical [17].

Inhibiting effect in vitro on calcium oxalate crystallization. After mixing 1 mL of calcium chloride solution (5 mmol/L) with 1 mL of distilled water to read the blank, we added 1 mL of sodium oxalate solution (7.5 mmol/L) to trigger crystallization. The optical density of the solution was measured at 620 nm for 10 min.

To determine the *in-vitro* effect of the propolis and bee pollen extracts on calcium oxalate formation, we mixed 1 mL of calcium chloride solution with 1 mL of each extract at 2, 4, 8, and 16 mg/mL. After adding 1 mL of sodium oxalate solution, the procedure was repeated with citric acid at 6 mM as a positive control [18]. The absorbance was recorded at 620 nm. The inhibition percentage (I, %) was calculated by the Eq. (3):

$$I = \frac{N_{Ss} - N_{Si}}{N_{Ss}} \times 100 \quad (3)$$

where N_{Si} and N_{Ss} were the nucleation slope test results with and without the inhibitor, respectively [18].

Interaction of xanthine dehydrogenase and phenolic compounds *in silico*. We used the *in-silico* method to study the interaction between xanthine dehydrogenase with rutin and liquiritin as the major phenolic compounds in the propolis and bee pollen extracts.

Protein preparation. The structure of xanthine dehydrogenase (ID: 1N5X at a resolution of 2.80 Å) was obtained from the Protein Data Bank at www.pdb.org. The Gasteiger-type atomic charges and hydrogen atoms were added to the xanthine dehydrogenase structure using the AutoDockTools (ADT) software. The modified protein structures were saved in the PDBQT format for subsequent docking studies.

Ligand preparation. Rutin and liquiritin were downloaded from the PubChem database in the SDF format and converted to the PDB format using the PyMOL software. To evaluate the energy of the system and facilitate the ligand binding, the protein receptor was enclosed in a three-dimensional grid encompassing the active site. The grid box allowed a free rotation of the ligand and was centered on the ligand with dimensions adjusted to fit all the ligands under study. The grid spacing was fixed at 1 Å.

Root mean square deviation. The AutoDock Vina software was used to determine the interactions between xanthine dehydrogenase and the phenolic ligands. The reliability of the docking program was assessed by calculating the root mean square deviation (RMSD) between the predicted ligand conformations and the experimental complex conformations. The maximal allowable difference in the root mean square deviation was 2 Å to ensure accuracy. Discovery Studio 4.0 made it possible to validate the accuracy of the docking predictions and the visual analysis.

Statistical analysis. The results were given as mean ± standard deviation (SD). The statistical analysis involved the SPSS software. The analysis of variance (ANOVA) revealed differences in mean values. The Tukey test highlighted statistically significant differences ($p < 0.05$).

RESULTS AND DISCUSSION

Bioactive content. The total phenolic content of the extracts under study varied from 41.25 ± 0.93 to 129.28 ± 1.51 mg GAE/g. The ethanolic extract of bee pollen had the highest total phenolic content (129.28 ± 1.51 mg GAE/g) ($p < 0.05$) followed by the methanolic extract of propolis (93.50 ± 2.65 mg GAE/g) and the ethanolic extract of propolis (93.16 ± 2.49 mg GAE/g). The lowest total phenolic content (41.25 ± 0.93 mg GAE/g) belonged to the methanolic extract of pollen ($p < 0.05$) (Table 1).

The statistical analysis revealed that the ethanolic extract of bee pollen had the maximal total flavonoid content (77.58 ± 1.95 mg QEQ/g) followed by the ethanolic extract of propolis (34.91 ± 3.25 mg QEQ/g). The lowest total flavonoid fraction was detected in the methanolic extract of bee pollen (10.54 ± 1.45 mg QEQ/g) (Table 1).

Nieva Moreno *et al.*, who studied the total phenolic and flavonoid contents of bee pollen extracts from different regions of Argentine, reported them as 19.28–48.90 mg GAE/g and 13.39–42 mg CEQ/g, respectively [19]. Ahn *et al.* reported that the total phenolic content of Chinese propolis varied from 85 ± 2 to 228 ± 8 mg GAE/g [12]. Moreira *et al.* defined the total phenolic content of methanolic propolis extract as ranging from 151.00 ± 0.01 to 329.00 ± 0.01 mg GAE/g [20]. Our results were much higher compared to those reported by Mohammadzadeh, who found that the total phenolic

Table 1 Bioactive content of propolis and bee pollen extracts

Extracts	Total phenolic content, mg GAE/g	Total flavonoid content, mg QEQ/g
Methanolic extract of propolis	93.50 ± 2.65^{b}	14.36 ± 1.82^{c}
Ethanolic extract of propolis	93.16 ± 2.49^{b}	34.91 ± 3.25^{b}
Methanolic extract of bee pollen	41.25 ± 0.93^{c}	10.54 ± 1.45^{d}
Ethanolic extract of bee pollen	129.28 ± 1.51^{a}	77.58 ± 1.95^{a}

Different letters in the same raw are significantly different ($p < 0.05$); mg GAE/g = 1 mg gallic acid equivalent per 1 g; mg CEQ/g = 1 mg catechin equivalent per 1 g

content of Iranian propolis extracts varied from 3.08 ± 0.02 to 8.46 ± 0.03 mg EAG/g [21]. Our results were also much higher than those published by Asem *et al.*, in whose study the total phenolic and flavonoid contents of Malaysian bee propolis extracts varied from 135.93 ± 5.95 to 326.10 ± 4.94 μM EAG/g and 28.57 ± 3.17 to 55.16 ± 7.52 μM QEQ/g, respectively [22].

Our findings were in accordance with those reported by Carpes *et al.*, who studied the total flavonoid content of Brazilian bee pollen (2.10–28.33 mg CEQ/g) [23]. According to Ahn *et al.*, the total flavonoid contents of Korean propolis extract ranged between 15.90 and 135.20 mg CEQ/g [24]. A recent study with 13 types of Anzer pollens from Turkey reported that the flavonoid contents ranged from 44.07 to 124.10 mg CEQ/g. The observed differences may be associated with the extraction conditions, the local vegetation, and the species of stingless bees [25].

High performance liquid chromatography (HPLC) showed the following list of major phenolic compounds: caffeic, syringic, ellagic, hydroxybenzoic, vanillic, as well as ferulic and o'coumaric acids [26–28]. Shi *et al.* and Rzepecka-Stojko *et al.* described gallic, benzoic, cinnamic, and phenylacetic acids as the dominant phenolic compounds in bee pollen [29, 30]. Luteolin, quercetin, rutin, formononectin, and liquiritin were the major flavonoids in Brazilian propolis [31, 32]. Apigenin, rutin, catechin, epicatechin, luteolin, quercetin, kaempferol, and naringenin were identified in bee pollen, and some of them could be antioxidant components [33, 34].

In-vitro* antioxidant potencies. *β-carotene-linoleic assay. Table 2 shows that the highest antioxidant capacity ($p < 0.05$) belonged to the ethanolic extract of propolis (63.05 ± 3.49%), whereas the ethanolic extract of bee pollen had the lowest antioxidant capacity (16.67 ± 1.08%). Based on a *β*-carotene bleaching assay, the order of antiradical capacity of the propolis and bee pollen extracts was as follows: bee pollen ethanolic extract < bee pollen methanolic extract < propolis methanolic extract < propolis ethanolic extract. None of the test samples exceeded the potency of butylated hydroxytoluene (96.35 ± 1.29%).

DPPH radical-scavenging. The ethanolic extract of bee pollen showed the highest level of antiradical efficiency (9.42 ± 0.0028 μg/mL) while the methanolic extract of bee pollen had the lowest one (33.56 ± 0.012 μg/mL). Based on the DPPH method, the order of antioxidant activity of extracts was as follows: bee pollen methanolic extract < propolis methanolic extract < propolis ethanolic extract < bee pollen ethanolic extract. No significant difference ($p > 0.05$) was detected between the antioxidant potencies of butylated hydroxytoluene and the ethanolic extract of bee pollen (Table 2).

A combination of more than one method is necessary to cover different mechanisms of antioxidant action, e.g., synergistic interactions. In this research, each extract was tested for its antioxidant activity by two complementary and universally methods, namely the *β*-carotenelinoleic acid system and the DPPH assay. Carpes *et al.* reported that ethanolic extracts of Brazilian bee pollen exhibited potent antioxidant activity by bleaching the *β*-carotene assay: the inhibition percentage varied from 40 to 90% [23]. According to Zhang *et al.*, caffeoylquinic acids and artepillin C were the major effective metabolites for quality control of Brazilian propolis due to their high antiradical capacity [34]. The ethanol extracts of propolis from different regions of Argentine showed antiradical capacity against DPPH, which varied from 20.00 ± 1.10 to 67.5 ± 2.3% at 20 μg/mL [16]. Gulcin *et al.*, who studied lyophilized aqueous extracts of Turkish propolis with the DPPH test, reported an IC_{50} of 31.81 μg/mL [35]. Khider *et al.* tested the antioxidant potency of the methanolic extracts of Egyptian bee pollen from maize, clover, and date palm using the DPPH assay [36]. The respective values of IC_{50} were 0.6, 0.8 and 0.7 μg/mL.

Table 2 Antioxidant activities of propolis and bee pollen extracts

Extracts	*β*-carotene bleaching assay, %	IC_{50} DPPH, μg/mL
Methanolic extract of propolis	20.35 ± 2.62[c]	11.80 ± 0.0043[b]
Ethanolic extract of propolis	63.05 ± 3.49[b]	10.95 ± 0.0068[b]
Methanolic extract of bee pollen	17.18 ± 1.90[c]	33.56 ± 0.012[a]
Ethanolic extract of bee pollen	16.67 ± 1.08[c]	9.42 ± 0.0028[c]
Butylated hydroxytoluene	96.35 ± 1.29[a]	4.59 ± 0.00086[c]

Different letters in the same raw are significantly different ($p < 0.05$)

Therefore, the antioxidant capacity of such bee by-products as propolis and bee pollen depends on their phenolic content.

Boeing *et al.* proved that phenolic components were responsible for the antioxidant activity of berry extracts [37]. In their case, methanol was the most efficient extraction solvent, followed by water and ethanol. This result could have been due to better solvation of antioxidant compounds present in fruits as a result of interactions (hydrogen bonds) between the polar sites of the antioxidant molecules and the solvent.

Antilithiasic potency of propolis and pollen extracts. *Crystallization kinetics of calcium oxalate.* The crystallization was significant ($p < 0.001$) in the presence of citric acid at 6 mM, compared to that of the blank, which contained no extract. The crystallization kinetics in the presence of the propolis and bee pollen ethanolic extracts at 8 and 16 mg/mL and in the presence of citric acid were not significantly different ($p > 0.05$). The absorbance values of citric acid proved to be significantly different ($p < 0.05$), compared to those of different concentrations of propolis and pollen ethanolic extracts (2, 4, 8, and 16 mg/mL). A significant difference ($p < 0.001$) was recorded between the absorbance of citric acid and those of the ethanolic extracts of propolis and bee pollen at 2 and 4 mg/mL (Figs. 2 and 3).

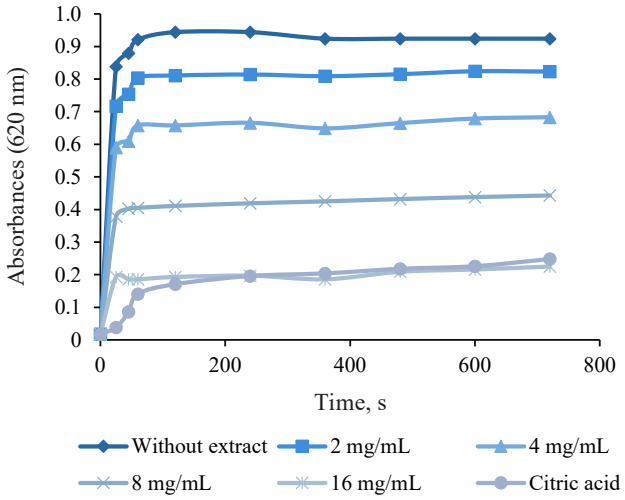


Figure 2 Inhibiting effect of ethanolic bee pollen extract on calcium oxalate crystallization

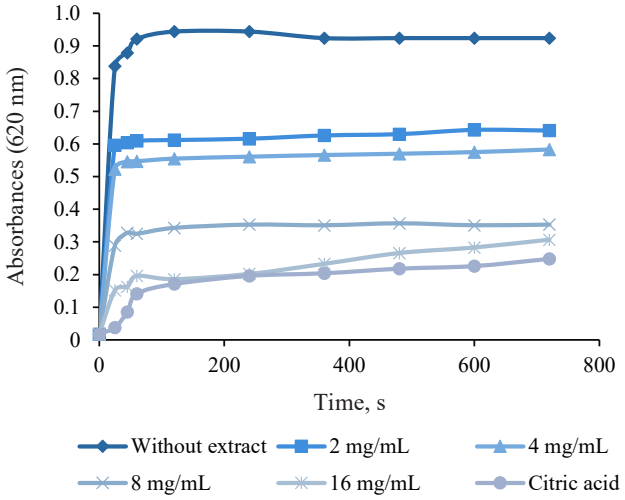


Figure 3 Inhibiting effect of methanolic bee pollen extract on calcium oxalate crystallization

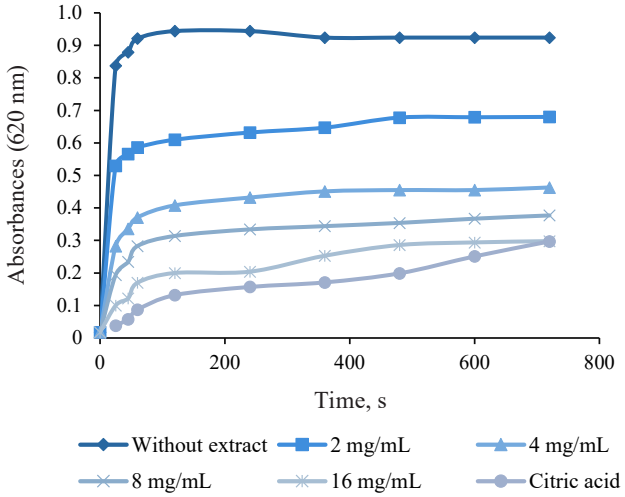


Figure 4 Inhibiting effect of ethanolic propolis extract on calcium oxalate crystallization

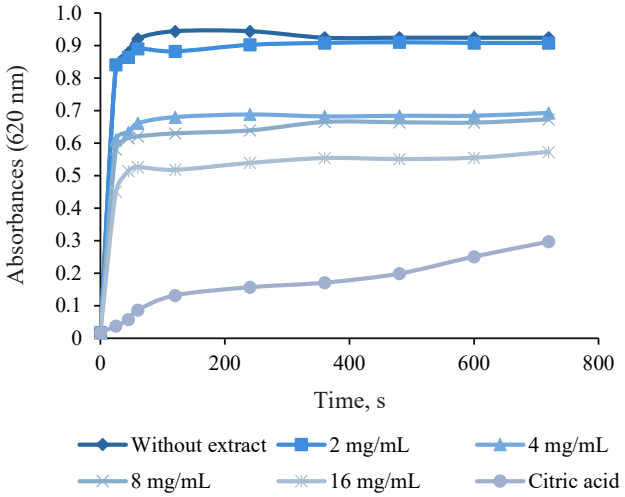


Figure 5 Inhibiting effect of methanolic propolis extract on calcium oxalate crystallization

The crystallization kinetics in the presence of the methanolic extract of pollen at 16 mg/mL did not differ significantly ($p > 0.05$) from that obtained in the presence of citric acid. However, a significant difference ($p < 0.05$) was recorded between the absorbance of citric acid ($p < 0.05$) and those of the methanolic extracts of propolis and pollen at 2, 4, 8, and 16 mg/mL. A very significant difference ($p < 0.01$) was recorded between the absorbances of citric acid and that of the methanolic extract of pollen at 2, 4, 8, and 16 mg/mL. The crystallization kinetics in the presence of citric acid at 6 mM proved to be very significant ($p < 0.001$), compared to that observed in the blank (without extract) (Figs. 4 and 5).

Inhibition percentage of calcium oxalate crystallization. The inhibition percentage (I, %) was calculated from turbidity slopes with and without propolis and bee pollen extracts. The inhibition percentage of the extracts was lower than that observed in citric acid (91.90 ± 0.26%). The latter showed significant antilithiasis activity ($p < 0.001$), compared to the ethanolic extract of propolis and the methanolic extract of pollen at 2, 4, 8, and 16 mg/mL. The inhibition percentage of citric acid crystallization was highly significant ($p < 0.01$), compared to those of the ethanolic propolis extract (82.83 ± 0.84%), the methanolic pollen extract (80.480 ± 0.021%), and the ethanolic pollen extract (80.480 ± 0.021%) at 16 mg/mL (Fig. 6).

Many studies report various effects of medicinal plant extracts on lithogenesis. Plants inhibit calcium oxalate crystallization under different protocols *in vivo* and *in vitro*. However, according to our knowledge, no scientific research ever evaluated the anti-lithiasic activity of propolis and bee pollen extracts. We compared our results with data on extracts from different plants reported by other authors.

Kachkoul *et al.* found a potent antilithiasic effect of *Ammi visnaga* L. and *Punica granatum* L., the respective inhibition percentages being 97.80 ± 0.12 and 73.25 ± 0.81%

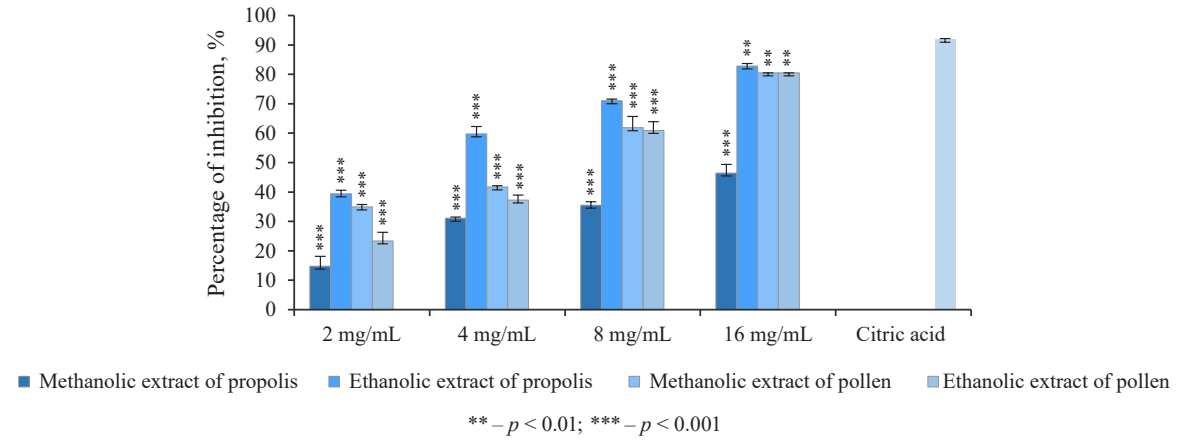


Figure 6 Percentage of crystallization inhibition in presence of extracts

Table 3 Binding free energy (ΔG), AutoDock Vina code

Ligands	Binding free energy (ΔG), kcal/mol
Rutin	–9.3
Liquiritin	–9.2

during nucleation and 83.46 ± 1.34 and 59.44 ± 3.30% during aggregation, respectively [38]. This inhibiting effect could be attributed to the abundance of bioactive molecules in these plants, e.g., polyphenols and flavonoids, which probably worked via their capacity to form soluble species to reduce the formation of oxalate stones.

The aqueous extract of *Cynodon dactylon* (L.) Pers. showed an antilithiasic effect with inhibition percentages of 60% at 1 mg/mL. Flavonoids exhibited an inhibitory effect on crystal growth by changing the crystal state and degree of hydration of calcium oxalate from the insoluble monohydrate form to poorly soluble dihydrate form and a decrease in numbers and sizes of aggregates [39]. Li *et al.* reported citrate as a potent inhibitor of the formation and crystallization of oxalate stones [40]. In their case, the presence of citric acid could modulate the water content of nucleated amorphous precipitates and hence promoted the formation of different calcium oxalate hydrates. Gallic acid altered the nucleation and the formation of calcium oxalate hydrates.

Khan *et al.* described the effect of *A. visnaga* seeds on kidney stones: the anti-calcination was mainly due to the strong diuretic activity and attenuation of hypersecretion and hyperbilirubinemia exerted by visnaga seeds [41].

Benalia *et al.* conducted an *in-vitro* study on dissolution of cystine stones using four traditional plant extracts and a saline control solution [42]. They subjected the calcinations to magnetic agitation for eight weeks. The plants examined were *Arenaria ammophila* L. (leaves and stems), *Parietaria officinalis* L. (leaves and flowers separately), and *Paronychia argentea* L. (flowers).

Interaction of xanthine dehydrogenase and phenolic compounds *in silico*. *Energy.* Rutin formed the most stable complex with xanthine dehydrogenase with a binding free energy (ΔG) of –9.3 kcal/mol, followed by liquiritin with –9.2 kcal/mol (Table 3).

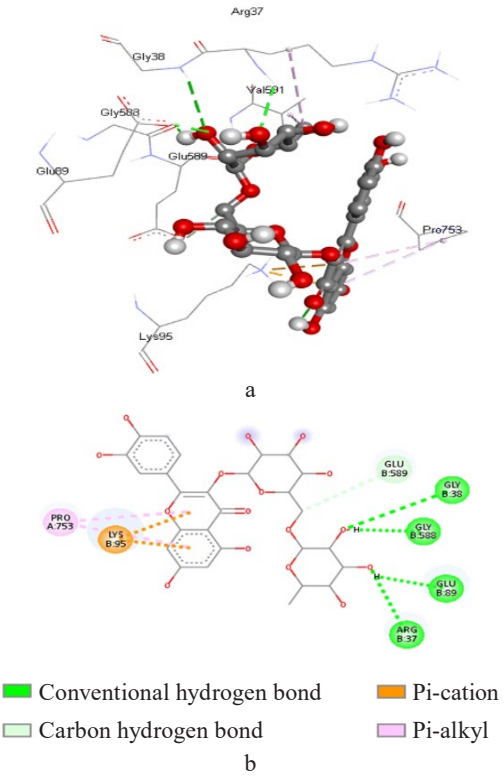


Figure 7 Three-dimensional (a) and dimensional (b) visualization of interactions between the active site of xanthine dehydrogenase and rutin

Interaction of ligands and xanthine dehydrogenase. *Interaction of rutin and xanthine dehydrogenase.* A three-dimensional visualization of the interactions between the active site of xanthine dehydrogenase and rutin showed that the interaction involved lysine 95, proline 753, glutamate 89, glycine 588, glycine 38, and glutamate 589 (Fig. 7).

According to the visualization (Discovery Studio), rutin penetrated well into the active site of xanthine dehydrogenase by forming different Pi-cation, Pi-alkyl, hydrogen, Pi-anion, and hydrogen carbon interactions (Table 4).

Interaction of liquiritin and xanthine dehydrogenase. A three-dimensional visualization of the interactions

Table 4 Interactions between rutin and xanthine dehydrogenase

Residue	Type of interaction	Distance (Å)
Lysine 95	Pi-cation	3.35
Proline 753	Pi-alkyl	5.50
Glutamate 89	Hydrogen	2.12
Glycine 588	Hydrogen	1.9
Glycine 38	Hydrogen	3.07
Glutamate 589	Carbon hydrogen bond	3.36

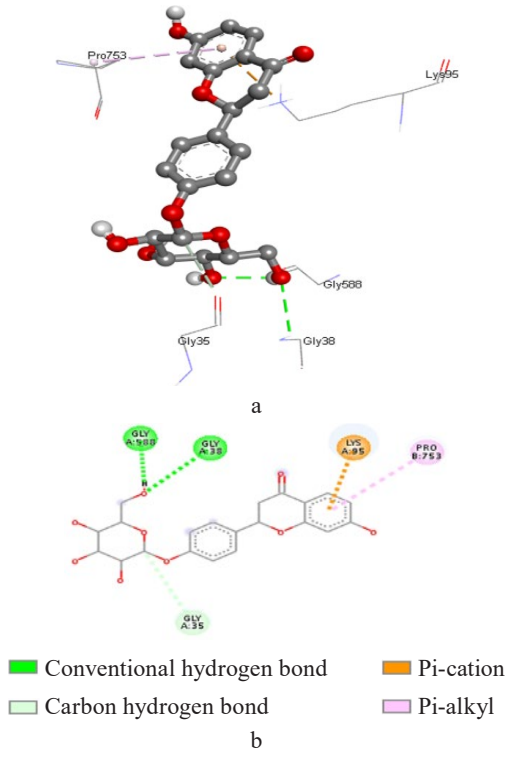


Figure 8 Three-dimensional (a) and dimensional (b) visualization of interactions between the active site of xanthine dehydrogenase and liquiritin

Table 5 Interactions between liquiritin and xanthine dehydrogenase

Residue	Type of interaction	Distance (Å)
Glycine 38, 588	Hydrogen	2.96
Glycine 588	Hydrogen	1.76
Lysine 95	Pi-cation	3.81
Proline 753	Pi-alkyl	5.34
Glycine 35	Carbon hydrogen bond	3.71

between the active site of xanthine dehydrogenase and liquiritin showed that the interaction involved glycine 38, glycine 588, glycine 35, lysine 95, and proline 753 (Fig. 8).

According to the visualization (Discovery Studio), liquiritin penetrated well into the active site of xanthine dehydrogenase by forming different Pi-cation, Pi-alkyl, hydrogen, Pi-anion, and hydrogen carbon interactions (Table 5).

Reliability testing of the molecular docking program. ***Root Mean Square Deviation.*** To predict the interaction between the ligands and the enzyme, we determined the exact position of each ligand in relation to its receptor. The root mean square deviation program compared the model and the structure of the reference ligand (co-crystallized). A root mean square deviation of ≤ 2 Å was considered acceptable [43]. In this study, xanthine dehydrogenase was the enzyme complex under analysis (PDB: 1n5x). After extracting the co-crystallized ligand from the target and repositioning it through docking in the active site, we calculated the root mean square deviation of the best position of the reference ligand after docking and compared it to its crystallographic binding mode before docking. The root mean square deviation value of hydroxytyrosol in the software-generated model stayed below 2 Å (Table 6).

Table 6 Root mean square deviation values of ligand interaction with xanthine dehydrogenase

Ligands	Root mean square deviation (Å)
Rutin	1.257
Liquiritin	1.839

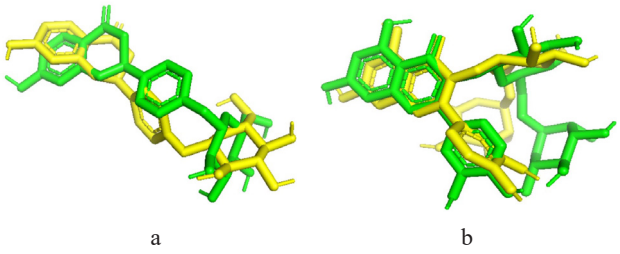


Figure 9 Superposition of inhibitors by Pymol to calculate root mean square deviation: (a) rutin, (b) liquiritin

Visual analysis plays a crucial role in complementing the numerical root mean square deviation values. When we examined the selected complexes of xanthine dehydrogenase (PDB: 1n5x) and ligands using PyMOL, the ligands predicted by AutoDock-Vina aligned well with the reference ligand. The visual confirmation substantiated the previously obtained values for root mean square deviation (Fig. 9).

Xanthine oxidase forms through the oxidation and/or proteolytic conversion of xanthine dehydrogenase. This enzyme occurs vascular cells, circulates in plasma, and binds to the extracellular matrix of endothelial cells. It facilitates the metabolism of nicotinamide adenine dinucleotide hydrogen (NADH), molecular oxygen, hypoxanthine, and xanthine, resulting in the production of O and HO, which makes it a significant source of reactive oxygen species [44]. Inhibiting this enzyme can protect against diabetic kidney disease and endometrial hyperplasia by reducing oxidative stress and improving uterine-reduced glutathione and superoxide dismutase, as well as inhibiting the expression of phosphatidylinositol-3-kinase (PI3K), Akt, and vascular endothelial growth factor (VEGF) [45, 46]. Antioxidants, such as phenolic acids and flavonoids, can inhibit xanthine oxidase activity. The method was validated by attaching

the inhibitory ligand to xanthine oxidase, with root mean square deviation values ranging from 1.019 to 2.35 [45]. Cinnamon phenolic acid inhibited xanthine dehydrogenase and oxidase [47]. Serrano *et al.* studied the inhibition mechanisms of eight structurally diverse phenolic compounds in fruits, i.e., quercetin, quercetin-3-rhamnoside, 4,5-O-dicaffeoylquinic acid, 3,5-O-dicaffeoylquinic acid, 3,4-O-dicaffeoylquinic acid, 4-O-caffeoylquinic acid, 3-O-caffeoylquinic acid, and caffeic acid [48]. They used the methods of 1H NMR spectroscopy, atomic force microscopy, and computational techniques to prove that these biomolecules were potent inhibitors of the enzyme. Amraoui *et al.* studied hydroxytyrosol as major phenolic compounds in *Olea europaea* L. leaves and revealed an effective xanthine dehydrogenase inhibition *in silico* [49].

The abovementioned studies prove that inhibiting xanthine oxidase with phenolic compounds can help prevent various diseases.

CONCLUSION

The research demonstrated appreciable levels of total phenolic and flavonoid contents in the ethanolic extract of such apicultural products as bee pollen and propolis. These natural compounds could be recommended as functional supplements in the treatment of various diseases, as well as natural sources of antioxidants in foods.

In our study, the ethanolic extract of propolis had notable inhibitory effects on the crystallization of calcium oxalate monohydrate. Based on the energy and root mean square deviations, the selected ligands were able to inhibit xanthine dehydrogenase. Bee pollen and propolis proved to contain components that can be used as a natural alternative to other methods of pharmaceutical prevention and treatment of kidney stones.

CONTRIBUTION

S. Ali Haimoud, A. Cheikh, S. Ghiboub, L. Ali Haimoud, K. Bahri, H. Benabed, B. Ayache, C. Beskri, and I. Boudali conceived and designed the analysis. S. Ali Haimoud, A. Cheikh, and S. Ghiboub collected the data. S. Ali Haimoud contributed data and analysis tools. L. Ali Haimoud, F. Arioui, and M. Medjekane performed the analysis. Ali Haimoud wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests related to the publication of this article.

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ORCID IDs
Safia Ali Haimoud https://orcid.org/0000-0002-6693-7942
Fatiha Arioui https://orcid.org/0000-0002-3160-5550
Meriem Medjekane https://orcid.org/0000-0002-7805-6499
Leila Ali Haimoud https://orcid.org/0009-0004-2819-9178
Khelifa Bahri https://orcid.org/0009-0003-0873-8632
Asma Cheikh https://orcid.org/0009-0004-0022-6322
Saadia Ghiboub https://orcid.org/0009-0008-3658-4948
Hanane Benabed https://orcid.org/0009-0008-1662-5765
Ikram Boudali https://orcid.org/0009-0003-6731-6894
Besma Ayache https://orcid.org/0009-0008-7483-6069
Chaima Beskri https://orcid.org/0009-0008-6906-5490