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By:
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In vitro and *in silico* study of the anti-hyperuricemia and anti-xanthine oxidase activity of some natural extracts and pure molecules

Defended in March 2023 to the jury composed of:

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Dedication

I dedicate my dissertation work to my family and many friends

A special feeling of gratitude

To my mother,

Who has been a source of encouragement and inspiration to me throughout my life, thank you for your unconditional love and prayers.

To my father,

Whose good examples have taught me to work hard for the things that I aspire to achieve.

To my beloved brother and sisters;

Who have been nicely my supporters until my research was fully finished, particularly my dearest sister, Hadjer, who stands by me when things look bleak.

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To all my family, the symbol of love and giving,

My friends who encourage and support me.

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Abstract

With lifestyle changes, gout has become common; excessive consumption of red meat and seafood can lead to hyperuricemia and then acute gout attacks. Recently, new strategies have been applied; using natural products and some approved synthetic products to treat the disease. We aim to evaluate *in vitro* and *in silico*, the inhibition activity with kinetics of four vitamins like: vitamin C, vitamin E, vitamin D3, and vitamin B9, three purified molecules: (harmaline and harmine) from *Peganum harmala* L., (hispidin) from *Inonotus hispidus* (Bull.) P.Karst., and two extracts: flavonoids (CEAE) and alkaloids (CAE) from *Cupressus sempervirens* L., to bovine milk xanthine oxidase (BXO), molecular docking using GOLD was done to explain the mechanism of action related to its inhibition and the pharmacokinetics (PK) parameters were checked to confirm their safety using preADMET 2.0 servers, the best-ranked inhibitors were chosen based on the approved PK properties and the best PLPchem score generated by GOLD. The results show that BXO have a K_m value of 163.55 µM with V_{max} of 37 U, the tested compounds present an important inhibition activity to BXO with an IC50 of 34.10 ± 0.21 µM (vitamin B9), 36.68 ± 1.50 µM (vitamin E), 39.01 ± 0.02 µM (vitamin C), and 100.28 ± 0.33 µM (vitamin D3), 48.52 ± 1.76 µM (harmine), 39.72 ± 0.32 µM (hispidin), and 51.00 ± 1.00 µM (harmaline), 3.52 ± 0.04 µg/ml (CAE), and 8.46 ± 1.98 µg/ml (CEAE), compared to the control (allopurinol with 32.03 ± 0.73 µM). The kinetic study shows that vitamins were non-competitive inhibitors with Ki values of 12 ± 1.41 µM (vitamin C), 29 ± 1.06 µM (vitamin E), 15 ± 1.76 µM (vitamin B9), and 20 ± 0.71 µM (vitamin D3), the purified molecules were non-competitive inhibitors such as harmaline (Ki = 11 ± 2.12 µM) and harmine (Ki = 2.5 ± 0.00 µM), while hispidin (Ki = 3.5 ± 0.00 µM) was a competitive inhibitor. The *in-silico* results show that lignan rhamnoside, hispidin, and vitamin E were the best inhibitors model with approved PK; the other molecules were saved as moderate to weak inhibitors with suitable PK properties. Eventually, the tested inhibitors could be significant in drug discovery, especially in treating gout; therefore, drug development, including clinical trials, should be done with these promising results.

Keywords: Bovine xanthine oxidase, Vitamins, Hispidin, Harmine, ADMET, GOLD, Gout.

Résumé

Avec les changements de mode de vie, la goutte est devenue courante ; la consommation excessive de viande rouge et de fruits de mer peut entraîner une hyperuricémie, puis des crises de goutte aiguës. Récemment, de nouvelles stratégies ont été appliquées, utilisant des produits naturels et certains produits synthétiques approuvés pour traiter la maladie. Notre objectif est d'évaluer *in vitro* et *in silico*, l'activité inhibitrice avec la cinétique de quatre vitamines comme : la vitamine C, la vitamine E, la vitamine D3, et la vitamine B9, trois molécules purifiées : (harmaline et harmine) de *Peganum harmala* L., (hispidin) de *Inonotus hispidus* (Bull.) P.Karst., et deux extraits : flavonoïdes (CEAE) et alcaloïdes (CAE) de *Cupressus sempervirens* L., contre la xanthine oxydase du lait bovin (XOB). L'amarrage moléculaire utilisant le logiciel GOLD a été réalisé pour expliquer le mécanisme d'action lié à son inhibition et les paramètres pharmacocinétiques (PC) ont été vérifiés pour confirmer leur sécurité en utilisant les serveurs preADMET 2.0, les inhibiteurs les mieux classés ont été choisis basé sur les propriétés PC approuvées et du meilleur PLPchem score généré par GOLD. Les résultats montrent que la XOB a une valeur K_m de 163,55 µM avec une V_{max} de 37 U, les composés testés présentent une importante activité inhibitrice contre la XOB avec une IC50 de 34,10 ± 0,21 µM (vitamine B9), 36,68 ± 1,50 µM (vitamine E), 39,01 ± 0,02 µM (vitamine C), et 100,28 ± 0,33 µM (vitamine D3), 48,52 ± 1,76 µM (harmine), 39,72 ± 0,32 µM (hispidin), et 51,00 ± 1,00 µM (harmaline), 3,52 ± 0,04 µg/ml (CAE), et 8,46 ± 1,98 µg/ml (CEAE), comparé au contrôle (allopurinol avec 32,03 ± 0,73 µM). L'étude de la cinétique montre que les vitamines étaient des inhibiteurs non-compétitifs avec des valeurs Ki de 12 ± 1,41 µM (vitamine C), 29 ± 1,06 µM (vitamine E), 15 ± 1, 76 µM (vitamine B9), et 20 ± 0,71 µM (vitamine D3), les molécules purifiées étaient des inhibiteurs non-compétitifs comme l'harmaline (Ki = 11 ± 2,12 µM) et l'harmine (Ki = 2,5 ± 0,00 µM), alors que l'hispidin (Ki = 3,5 ± 0,00 µM) était un inhibiteur compétitif. Les résultats *in silico* montrent que la lignan rhamnoside, l'hispidin, et vitamine E étaient les meilleurs modèles inhibiteurs avec une pharmacocinétique approuvée ; les autres molécules ont été enregistrées comme inhibiteurs modérées à faibles avec des propriétés pharmacocinétiques convenable. Finalement, les inhibiteurs testés pourraient être importants dans la découverte de médicaments, en particulier pour le traitement de la goutte ; par conséquent, le développement de médicaments comprenant les essais cliniques, devrait être réalisé avec ces résultats prometteurs.

Mots clés : Xanthine oxydase bovine, Vitamines, Hispidin, Harmine, ADMET, GOLD, Goutte.

الملخص

انتشر مرض النقرس مؤخرا مع تغير أسلوب الحياة، يمكن أن يؤدي الاستهلاك المفرط للحوم الحمراء والمأكولات البحرية إلى ارتفاع نسبة حمض اليوريا ومن ثم نوبات حادة من النقرس. نشهد حديثا انه تم تطبيق استراتيجيات جديدة، باستخدام منتجات طبيعية وبعض المنتجات الاصطناعية المعتمدة لعلاج المرض. نهدف إلى تقييم النشاط التثبيطي مخبريا وفي السيليكون، إضافة إلى الحركية لأربعة فيتامينات مثل: فيتامين ج، فيتامين ه، فيتامين د 3، وفيتامين ب 9، ثلاثة جزيئات نقية معزولة: (حارمالين وحارمين) من الحرمل، (هيسبيدين) من سرة البطم، ومستخلصان: الفلافونويد والقلويدات من السرو، ضد انزيم زانثيدين أوكسيداز المستخرج من حليب البقر. تم الإرساء الجزيئي باستخدام برنامج (قولد) لشرح آلية العمل المتعلقة بالتثبيط وتم التحقق من خصائص حركيات الدواء (ح د) للتأكد من سلامتها باستخدام موقع أدميت أولي 2.0، وتم اختيار أفضل مرتبة بناءا على خصائص ح د امنة وأفضل نتيجة طاقة تحصلنا عليها من برنامج (قولد). وتبين النتائج أن قيمة ثابت ميكايلس للأنزيم بلغ 163.55 ميكرو مولار مع سرعة قصوى بلغت 37 وحدة، تمثل المركبات المختبرة نشاط تثبيط هاما للأنزيم المدروس مع تركيز مثبط نصفي البالغ 34.10 ± 0.21 ميكرو مولار (فيتامين ب 9)، و36.68 ± 1.50 ميكرو مولار (فيتامين ه)، و39.01 ± 0.02 ميكرو مولار (فيتامين ج)، و100.28 ± 0.33 ميكرو مولار (فيتامين د 3)، 48.52 ± 1.76 ميكرو مولار (حارمين) و39.72 ± 0.32 ميكرو مولار (هيسبيدين) و51.00 ± 1.00 (حارمالين) و3.52 ± 0.04 مغ/مل و8.46 ± 1.98 مغ/مل، بالمقارنة مع الشاهد (ألوبيرينول مع 32.03 ± 0.73 ميكرو مولار). تبين الدراسة الحركية أن الفيتامينات كانت مثبطات غير تنافسية ذات قيم ثابت تثبيط تبلغ 12 ± 1.41 ميكرو مولار (فيتامين ج)، و29 ± 1.06 ميكرو مولار (فيتامين ه)، و15 ± 1.76 ميكرو مولار (فيتامين ب 9)، و20 ± 0.71 ميكرو مولار (فيتامين د 3)، وكانت الجزيئات النقية مثبطات غير تنافسية مثل الحرمالين (ثابت تثبيط = 11 ± 2.12 ميكرو مولار) والحارمين (ثابت تثبيط = 2.5 ± 0.00 ميكرو مولار)، الهسيبيدين (ثابت تثبيط = 3.5 ± 0.00 ميكرو مولار) كان مثبطا تنافسيا. وتبين نتائج التجارب في السيليكون أن ليغنان رامنوسيد، والهسبيدين، والفيتامين ه هي أفضل النماذج المثبطة التي تعتمد على ح د امنة؛ اما بالنسبة للجزيئات الأخرى فقط سجلنا ان تثبيطها كان من معتدل الى ضعيف مع خصائص ح د مناسبة. ومن أفاق هذا العمل ان تتم دراسة المثبطات المختبرة وتطويرها من اجل انتاج العقاقير لمعالجة مرض النقرس بعد إجراء التجارب السريرية اللازمة.

كلمات مفتاحية: إنزيم زانثيدين أوكسيداز، فيتامينات، هيسبيدين، حارمين، أدميت، برنامج (قولد)، مرض النقرس.

List of scientific works completed during PhD thesis

International publications (08)

1. **Abderahmane LINANI**, Khedidja BENAROUS, Leila BOU-SALAH, Mohamed YOUSFI. Hispidin, Harmaline, and Harmine as potent inhibitors of bovine xanthine oxidase: Gout treatment, *in vitro,* ADMET prediction, and SAR studies. *Bioorganic Chemistry* 112(5):104937.
2. **Abderahmane LINANI,** Khedidja BENAROUS, Leila BOU-SALAH, Mohamed YOUSFI. The inhibitory kinetics of vitamins B9, C, E, and D3 on bovine xanthine oxidase: Gout treatment. *Chemico-biological Interactions 359(11):109922*.
3. **Abderahmane LINANI,** Talia SERSEG, Khedidja BENAROUS, Leila BOU-SALAH, Mohamed YOUSFI, Mohammed Nabil ALAMA, Md Ashraf GHULAM. *Cupressus sempervirens* L. flavonoids as potent inhibitors to xanthine oxidase: *in vitro*, molecular docking, ADMET and PASS studies. *Journal of biomolecular structure and dynamics*
4. **Abderahmane LINANI,** Khedidja BENAROUS, Leila BOU-SALAH, Mohamed YOUSFI, Souraya GOUMRI-SAID. Exploring Structural Mechanism of COVID-19 Treatment with Glutathione as a Potential Peptide Inhibitor to the Main Protease: Molecular Dynamics Simulation and MM/PBSA Free Energy Calculations Study. *International Journal of Peptide Research and Therapeutics 28(55):16.*
5. Lozan Todorov, Luciano Saso, Khedidja BENAROUS, Maria Traykova, **Abderahmane LINANI,** Irena Kostova. Synthesis, Structure and Impact of 5-Aminoorotic Acid and Its Complexes with Lanthanum (III) and Gallium (III) on the Activity of Xanthine Oxidase. *Molecules 26(15):4503*.
6. Leila BOU-SALAH, Khedidja BENAROUS, **Abderahmane LINANI**, Isabelle BOMBARDA, Mohamed YOUSFI. *In vitro* and *in silico* inhibition studies of five essential oils on both enzymes human and bovine xanthine oxidase. *Industrial Crops and Products* 143 (2020) 111949.
7. Khedidja BENAROUS, Leila BOU-SALAH, **Abderahmane LINANI**, Mohamed YOUSFI, Luciano SASO, Irena KOSTOVA. Lanthanide (III) complexes of bis-coumarins as strong inhibitors of bovine xanthine oxidase - molecular docking and SAR studies. Journal of Biomolecular Structure and Dynamics (2020).
8. BOU-SALAH Leila, BENAROUS Khedidja, **LINANI Abderahmane**, RABHI Faiza, CHAIB Kheira, CHINE Imane, BENSAIDANE Hadjer and YOUSFI Mohamed. Anti-inflammatory drugs as new inhibitors to xanthine oxidase: *in vitro* and *in silico* approach. Molecular and Cellular Probes 58 (2021): 101733

List of scientific works completed during PhD thesis

International Communications (02)

1. **Abderahmane LINANI**, Khedidja BENAROUS, Leila BOU-SALAH, et Mohamed YOUSFI. *In vitro* and *in silico* inhibition study of the garlic essential oil against human xanthine oxidase: gout treatment. Third International Symposium: Medicinal Plants and Materials (MPM-2020): Tebessa, Algeria.
2. Leila BOU-SALAH**,** **Abderahmane LINANI**, Khedidja BENAROUS, et Mohamed YOUSFI. Études *in silico* et *in vitro* de l'effet inhibiteur des huiles essentielles de *Pimpinella anisum* L. et de *Carum carvi* L. sur la xanthine oxydase humaine et bovine : traitement de la goutte. Third International Symposium: Medicinal Plants and Materials (MPM-2020): Tebessa, Algeria.

National Communications (02)

1. **Abderahmane LINANI**, Khedidja BENAROUS, Leila BOU-SALAH, et Mohamed YOUSFI. Potent gout treatment using ginger and camphor essential oils: *in vitro* and *in silico* inhibition study. Chimie analytique, matériaux et substances naturelles, Décembre 2019.
2. Leila BOU-SALAH, **Abderahmane LINANI**, Khedidja BENAROUS, et Mohamed YOUSFI. Évaluation *in vitro* et *in silico* de l'activité inhibitrice de l'huile essentielle de *Coriandrum sativum* L. sur la xanthine oxydase humaine et bovine**.** Chimie analytique, matériaux et substances naturelles, Décembre 2019.

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List of Abbreviations

A-D	L-N
Å : Angstrom **B.C :** Before Christ **Co A:** Coenzyme A **DED:** Double enzyme detection **DMSO :** Dimethyl sulfoxide **DOAJ :** Directory of Open Access Journals **DNA:** Deoxyribonucleic acid **DSV:** Discovery studio visualizer	**LDL :** Low density lipoprotein **Mo :** Molybdenum **NHANES :** National Health and Nutrition Examination Survey **NMR:** Nuclear magnetic resonance
E-H	**O-Q**
EDTA : Ethylene diamine tetra acetic acid **EC:** Enzyme Commission **GOLD:** Genetic Optimization for Ligand Docking **GLUT9:** Glucose transporter 9 **HDL:** High density lipoprotein **HHANES:** Hispanic Health and Nutrition Examination Survey **HPLC:** High-performance liquid chromatography	**OAT4 :** Organic anion transporter **PCOS:** Polycystic ovary syndrome **PK:** Pharmacokinetics **PDB :** Protein Data Bank
I-K	**R-X**
K_2HPO_4 : Dipotassium hydrogen phosphate **KH_2PO_4 :** Potassium dihydrogen phosphate	**RMSD :** Root Mean Square Deviation **RNA:** Ribonucleic acid **MS:** Mass Spectroscopy **SDF:** Spatial Data File **USDA:** United States Department of Agriculture **UA :** Uric acid **URAT1 :** Urate transport 1 **XO :** Xanthine oxidase **XOR :** Xanthine oxidoreductase

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Introduction

Gout or disease of kings is the oldest joint disease known to humans, first described in 2640 B.C., a form of arthritis known by the accumulation of excess serum uric acid (UA) crystals in joint and articulations and cause inflammation, the signs and symptoms occur suddenly, and often at night, it has characterized by severe painful attacks **[1]**. UA is a waste product of purines metabolism, particularly adenine and guanine, which are the precursors of nucleic acids DNA and RNA, reflecting the metabolic state of the body and have other important functions in the cell such as contribute to modulate energy metabolism, signal transduction, enters in the physiology of platelets, muscles and neurotransmission **[2]**.

It is catalyzed by the xanthine oxidoreductase enzyme (XOR) to its final form as UA, except in mammals it converts to allantoin by an enzyme called uricase that can be easily eliminated through urine. UA can be increased through exogenous and endogenous pathways such as excessive intake of high-purine and high-fructose, like consuming alcohol, high fats, high-refined carbohydrate, overeating meat and seafood's; it is mainly affected by endogenic pathway such as blood acidification, liver and small intestine catabolism **[3]**.

Approximately two-thirds of UA is excreted by the kidneys, while one-third is excreted by the gastrointestinal tract, the *Urate transporter 1* (URAT1) is the main transporter responsible of the reabsorption of UA inside renal tubules, *Glucose transporter 9* (GLUT9) and *Organic anion transporter 4* (OAT4) are the main UA-secreting transporters, however, their dysfunctions is rare; it causes urate transport disorders and cause hyperuricemia and gout **[4]**.

High serum UA levels is likely to induce a series of health issues like kidney disease, hypertension, endothelium injury, atherosclerosis, coronary artery disease, cardiovascular disease, decrease infertility by inhibiting oocyte meiosis **[5]** and metabolic syndromes **[6]**.

Some literature reports that in some patients UA tends to rise to higher levels, such as polycystic ovary syndrome (PCOS) since it can be found in the follicular fluid of the female ovary **[7, 8]**. Rotterdam **[9]** and NHANES I **[10]** studies suggested that there is a causal link

between hyperuricemia and myocardial infarctions and even death. The males were found to be more exposed than women **[11]**; often the main risk factors are diabetes, hypertension, chronic renal failure, reproductive hormones, obesity, sex **[12]**, age, family history of gout, medications like thiazide, diuretics, cyclosporine, and low-dose aspirin (<1000 mg per day) **[13]**. Generally, uric acid (UA) levels over 7 mg/dl in males and over 6 mg/dl in females are considered as hyperuricemia in a clinical aspect **[14]**.

The given drugs are the anti-inflammatories, especially in the first inflammation pics. Colchicine **[15]** and allopurinol **[16]** in chronic gout, the latter is competitive inhibitors to XO. However, these drugs are found dangerous, and lead to developing "the allopurinol hypersensitivity syndrome"**[17]**, which causes fever and rash; these medications, in general, affect the renal **[18]** and hepatitis by changing the liver function **[19]** and cause death **[20]**. Colchicine has been reported responsible for side effects like "nausea, vomiting, abdominal pain and diarrhea"**[21]**, it can cause man infertility **[22]**.

We headed to herbal medicine supported with a new strategy using vitamins to track down an effective treatment with lower side effects **[23]**. *Cupressus sempervirens* L., *Inonotus hispidus* (Bull.) P.Karst., and *Peganum harmala* L., plants are reported to have multiple activities like antiseptic, antiviral, anti-inflammatory, and diuretic **[24]**. Mostly, vitamins are known to improve immunity and maintaining the neurological system such as, vitamin C and vitamins B Group **[25, 26]**, especially folic acid (vitamin B9) **[27, 28]**.

This thesis aimed to evaluate the inhibition activity of some vitamins and plants secondary metabolites such as flavonoids and alkaloids extracts with its three pure molecules to the bovine milk xanthine oxidase (BXO) extract *in vitro* and *in silico* based on the double enzyme detection (DED) method, and then study the enzyme kinetics to determine the related constant V_{max}, K_m, and Ki and further the inhibition type. We supported this work by predicting the pharmacokinetics (PK) properties of our studied molecules as the oral administration

bioavailability and the ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties, using preADMET 2.0 server. To understand the biochemical mechanism of the used molecules to BXO and the preferred orientations with the catalytic amino acids inside the active site, a structure-activity relationship (SAR) study was performed using molecular docking with GOLD v4.1.2 and Discovery studio visualizer v20.0 (DSV) packages.

The manuscript is presented in the following way:

The first part presents a brief literature review where the general concepts gathering the essential information for this study are described. First, we describe the history of publications on xanthine oxidase from different points such as available sources of structures and published inhibitors. Second, we present the publications related to vitamins and xanthine oxidase from a statistical view, and then we describe the benefits of using vitamins and their therapeutic effects. Finally, we describe the plants used in this thesis, their traditional local use, their classifications and their therapeutic effects.

The second part, is devoted to the experimental section, where we listed all the materials and methodologies used during the work. Then we present the obtained results to be discussed furthermore in detail.

Lastly, we will end this manuscript with a conclusion and research perspectives.

Literature Review

I. Xanthine oxidase

1. Definition

The nucleotidase are responsible of producing nucleosides, which further converted to purine bases by nucleoside phosphorylase enzyme **[29]**. Among these purine bases, hypoxanthine and xanthine which are produced by the xanthine oxidase (XO), uric acid (UA) represents the final products in the catabolism of purines, it is further produced by the action of XO with a variety of enzymes that involved in its synthesis, eventually, XO is reported the main responsible enzyme in this process **[30]**.

2. Role in oxidative stress

Xanthine oxidase (figure 1) catalysis the oxidation of hypoxanthine to xanthine and then to UA, and plays an important role in reactive oxygen species (ROS) and superoxide radical production it has two convertible forms: xanthine dehydrogenase (XDH) and XO. XDH can convert NAD^+ to NADH via $FADH_2$, while XO utilize molecular oxygen (O_2) to produce UA, active superoxide ions (O^{-2}), and hydrogen peroxide (H_2O_2) via FAD **[2, 30]**. ROS proved diffuse into the cell cytoplasm and alter the normal function of mitochondria, affect lipid synthesis and lipid oxidation distortion leading to oxidative stress in the body **[31]**.

3. Hyperuricemia and gout disease

Xanthine oxidase was reported responsible for hyperuricemia **[32]**. High production of UA in the body due to the over consumption of red meats and sea foods or the inability of the kidneys to excrete it, leads to abnormally accumulated in body fluids in form of urate crystals; the latter causes immune system response; which stimulate prostaglandins and leukotrienes secretion, then cause the known joints inflammation (pain and swelling) which called gout attack **[33]**. Recently, published epidemiological studies have identified hyperuricemia as an independent risk factor for chronic kidney, cardiovascular, and hypertension diseases **[34]**. New studies on the pathogenesis of gout are currently in progress.

4. Structure

In this thesis, we used bovine xanthine oxidase (BXO) as the enzyme model for the *in vitro* assays, as reported in our previous work **[35]**. Initially, the BXO crystal structures (figure 1) in complex with the physiological substrate hypoxanthine, it is composed of 2439 residues with three chains named A, B, and C with a total molecular weight of 273.91 kDa **[36]**.

This enzyme holds five co-crystallized ligands like:

- Flavin-Adenine Dinucleotide (FAD),
- phosphonicacidmono-(2-amino-5,6-dimercapto-4-oxo-3,7,8a,9,10,10a-hexahydro-4h-8-oxa-1,3,9,10-tetraaza-anthracen-7-ylmethyl) ester (MTE),
- FE2/S2 (inorganic) cluster (FES),
- Di-oxothio-Molybdenum (vi) ion (MOS),
- Hypoxanthine (HPA);

The chain C hold the active site which composed of Glu802 Arg880, Ala910, Phe914, Phe1009, Thr1010, Ala1078, Ala1079, and Glu1261 **[36]**.

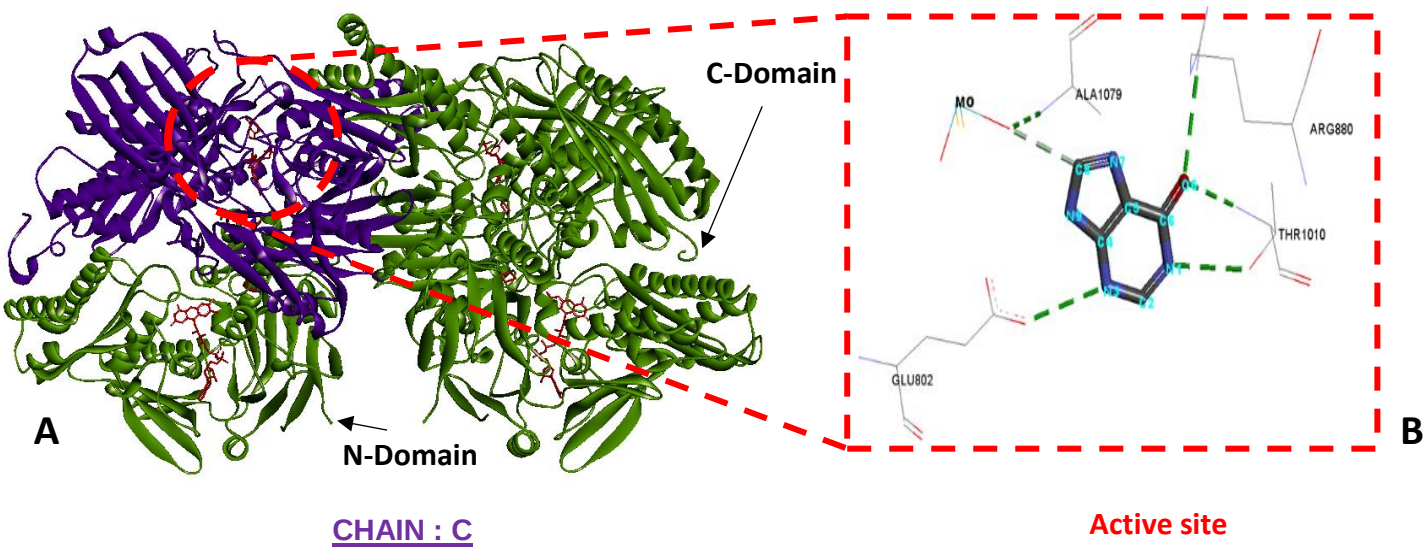


Figure 1. 3D structure of the bovine milk xanthine oxidase (PDB ID: 3nrz) complexed with hypoxanthine (HPA) in chain C, A: the 3D structure in helices mode and colored in green, chain C presented in helices and colored in purple, B: zooming on the active site colored in red, the ligand HPA presented in sticks and colored in default atom color.

5. Reaction mechanism

The catalysis is activated by a nucleophilic attack via the molybdenum center Mo (VI) Mo-OH on the C-8 carbon of the xanthine and in the presence of the catalytic amino acid Glu1261, which simultaneously leads to the reduction of Mo (VI) to Mo (IV). The first step consists in a reoxidation of the molybdenum center by electron transfer to Glu1261 of the active site, accompanied by the protonation of the Mo-SH bond, the second step consists in the deprotonation of the Mo-SH bond to Mo=S, the third step consists in regenerating the Mo-OH group of the molybdenum system (Mo (VI)) and releasing a new product which is the uric acid, consequently Glu1261 returns to its original ionic form **[36]**. Finally, the fourth step will repeat the cycle (figure 2).

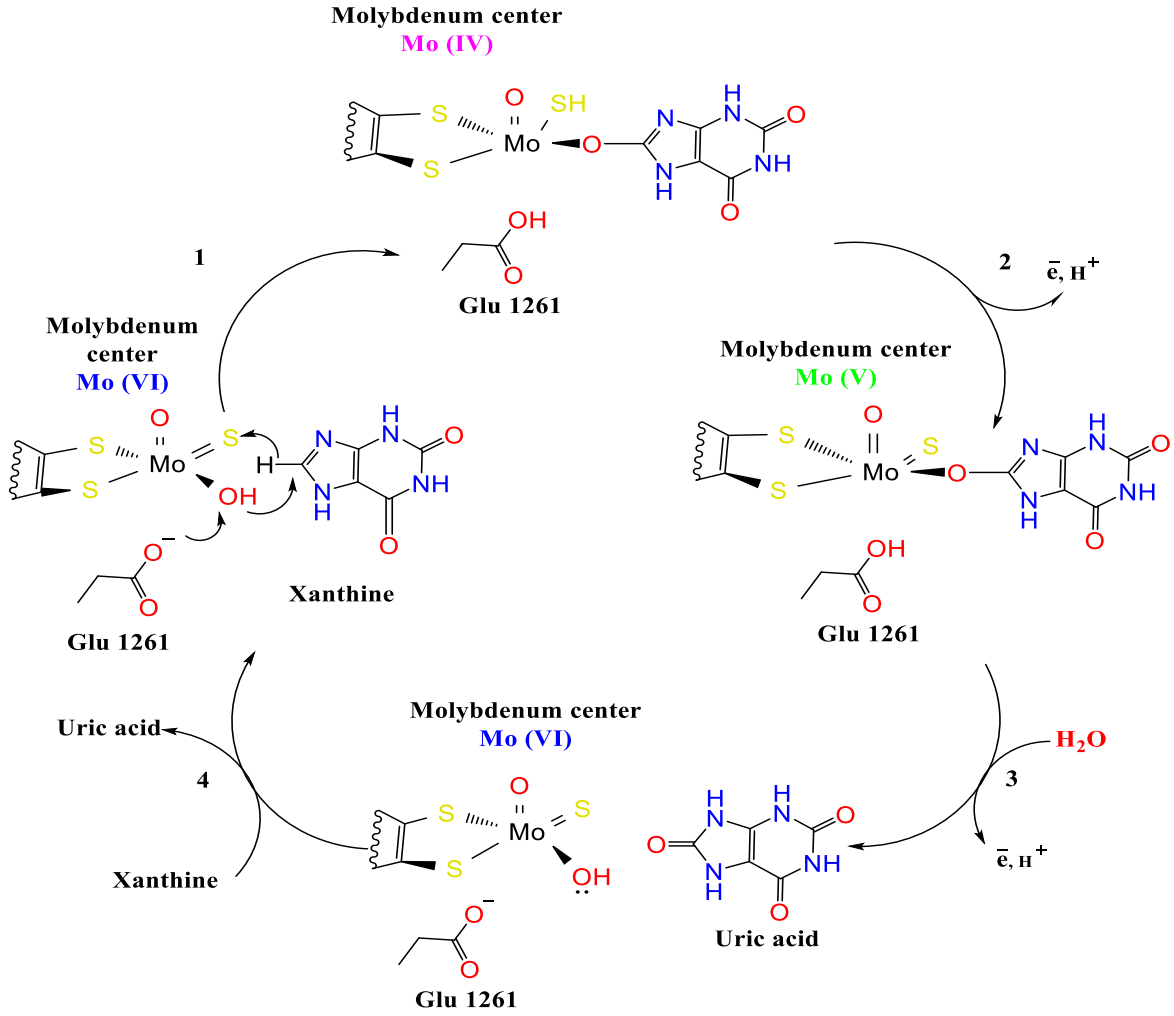


Figure 2. Scheme of the catalytic mechanism of xanthine oxidase **[36]**.

6. Previous works

Since its discovery by Morgan, Stewart and Hopkins in 1922, xanthine oxidase studies begun in 1925, the first study conducted by Malcolm Dixon **[37]**, the study consists of testing the destructive effect of hydrogen peroxide toward the XO when the purine bases are oxidized by molecular oxygen in presence of xanthine oxidase as a catalyst. In 1960s, studies on its genetics, structure, and function in various sources like liver and milk was achieved. After 1970, many studies conducted its isolation, purification, and types **[38]**.

Studies on the XO activation and inhibition from multiples sources was reported effective **[39-42]** like the indolinedione–coumarin hybrid molecules **[43]**, Thiazole-5-carboxylic acid derivatives **[44-46]**, naphthoflavones **[47]**, and plant secondary metabolites derivatives. The table below summarizes the important studies in this context.

Table 1. Some previous studies on xanthine oxidase.

Trials No.	Period	No of volun/pati	Affiliation/ Country	Theme research	Age (years)	Control	Studies conclusion	Meta-analysis
XO purification								
119	1939-2021	/	Different countries/ International	Xanthine oxidase purification from liver, milk, intestine, and bacteria	/	/	Pure xanthine oxidase	**[38]**
XO role in diseases								
40	2001-2010	33 867	Europe USA Argentina Canada	Treatment of cardiovascular disease	69 (Mean)	Allopurinol Oxypurinol	Improves endothelial function and circulating markers of oxidative stress	**[48]**
06	2014-2016	773	Europe Asia USA	Improving renal function	55 (Mean)	Febuxostat Allopurinol	Reduce chronic kidney disease	**[49]**
09	2010-2013	146 236	Europe China Korea	Atrial fibrillation	56-67	Placebo Allopurinol	Decrease atrial fibrillation	**[50]**
01	1985-2013	2 769	Europe	Xanthine oxidase gene variants	40 (Mean)	Placebo Allopurinol	Genetic variation in XOR lead to hypertension	**[51]**

XO natural inhibitors								
13	2006-2020	/	Asia Europe	Inhibition effect of flavonoids from leaves of *Perilla frutescens*	/	Febuxostat Allopurinol	Reduce XO activity (Flavanone Chalcone Aurone)	**[52]**
15	2005-2021	/	Different countries/ International	Inhibition effect of flavonoids from leaves of *Blumea balsamifera*	/	Febuxostat Allopurinol	Reduce XO activity (Flavonols Flavones Flavanones Dihydroflavonol derivatives)	**[53]**
01	2016	/	Puerto Rican	Inhibition effect of flavonoids from leaves of *Syzygium malaccense*	/	Allopurinol	Reduce XO activity (Ethanol extracts)	**[54]**
03	2012-2019	/	Algeria Poland Khuzestan	Inhibition effect of flavonoids from aerial part of *Capparis spinosa L*	/	Allopurinol	Reduce XO activity (Ethyl acetate) extract	**[55]**
01	2019	/	China	Inhibition effect of alkaloids from aerial parts of *Tabernaemontana bufalina*	/	Allopurinol	Reduce XO activity (Apparicine: indole alkaloid)	**[56]**
01	2009	/	Pakistan	Inhibition effect of alkaloids from aerial parts of *Isatis costata*	/	Allopurinol	Reduce XO activity (Costinones Isatinones Indirubin Trisindoline)	**[57]**
01	2017	/	Indonesia	Inhibition effect of alkaloids from *Peperomia pellucida*	/	Allopurinol	Reduce XO activity (Alkaloid extracts)	**[58]**
01	2000	/	Belgium Congo	Inhibition effect of alkaloids from aerial parts of *Cryptolepis sanguinolenta*	/	Allopurinol	Reduce XO activity (11-hydroxycryptolepine)	**[59]**
XO synthesized inhibitors								
09	2015-2021	/	Bulgaria Algeria	Inhibition effect of metallo-organic complexes	/	Allopurinol	Reduce XO activity (Complexes of bis coumarins)	**[60]**
03	2008-2020	/	China Ukraine	Inhibition effect of Schiff base transition metal complexes	/	Allopurinol	Reduce XO activity (Schiff base transition metal complexes)	**[61]**

No: number **volun/pati:** volunteer/patient

II. Vitamins

Humans require essential macronutrients such as proteins, healthy fats, carbohydrates, and micronutrients like minerals, vitamins, and water. Vitamins are organic compounds and essential micronutrients found in natural foodstuffs, its requirement needed in small amounts for humans; generally below 100 mg/day, vitamins play an important role in the body and have many health benefits; including tissue maintenance, bone and tooth formation, as a cofactor for enzymes, the vitamins reported essential for the fetal growth and development, as well as other biochemical and physiological functions in the body **[62]**.

Vitamins participate in metabolism and cell regulation processes like vitamin A **[63]**, it helps resist infections (vitamin C) **[64]**, react as antioxidants (vitamin E and C) **[65]**, improve the blood circulation and maintain the neurological system (vitamin B family) **[66]**, provides a hormone-like function (vitamin D), and helps generate energy from food **[67]**.

1. Vitamins classifications

There are 13 essential vitamins such as vitamin A, C, D, E, K, and the B vitamins group like thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), biotin (B7), pyridoxine (B6), cobalamin (B12), and folate (B9) **[68]**. It is recognized to play an important role in human health. According to their solubility, these vitamins can be divided into fat-soluble vitamins like A, D, E and K and water-soluble vitamins like vitamin B complex and vitamin C.

1.1.Fat-soluble vitamins

Fat-soluble vitamins are soluble in fatty tissue and can be stored in the body mainly in the liver, consumption of healthy fats helps to absorb these vitamins from the intestinal tract; generally, it should be taken weekly or monthly because it stays longer in the body stores and may last for months to years before developing health issues **[69]**.

1.1.1. Vitamin A

Vitamin A is a family of micronutrient in form of retinol, retinal, and retinoic acid (figure 3); the latter reported to have the most active biological activity **[70]**. Vitamin A presented as unsaturated structure contain monohydric alcohols that contain an alicyclic ring, known for its anti-inflammation proprieties and maintaining vision by regenerate the visual chromophore of rhodopsin for receiving light **[71]**; it can be included in the treatment of various infectious diseases and has a critical role in enhancing and regulate the immune system **[72]**, protecting epithelium which acts as the front line defense against pathogen invasion, preserve mucus integrity, promoting growth and development **[70]**.

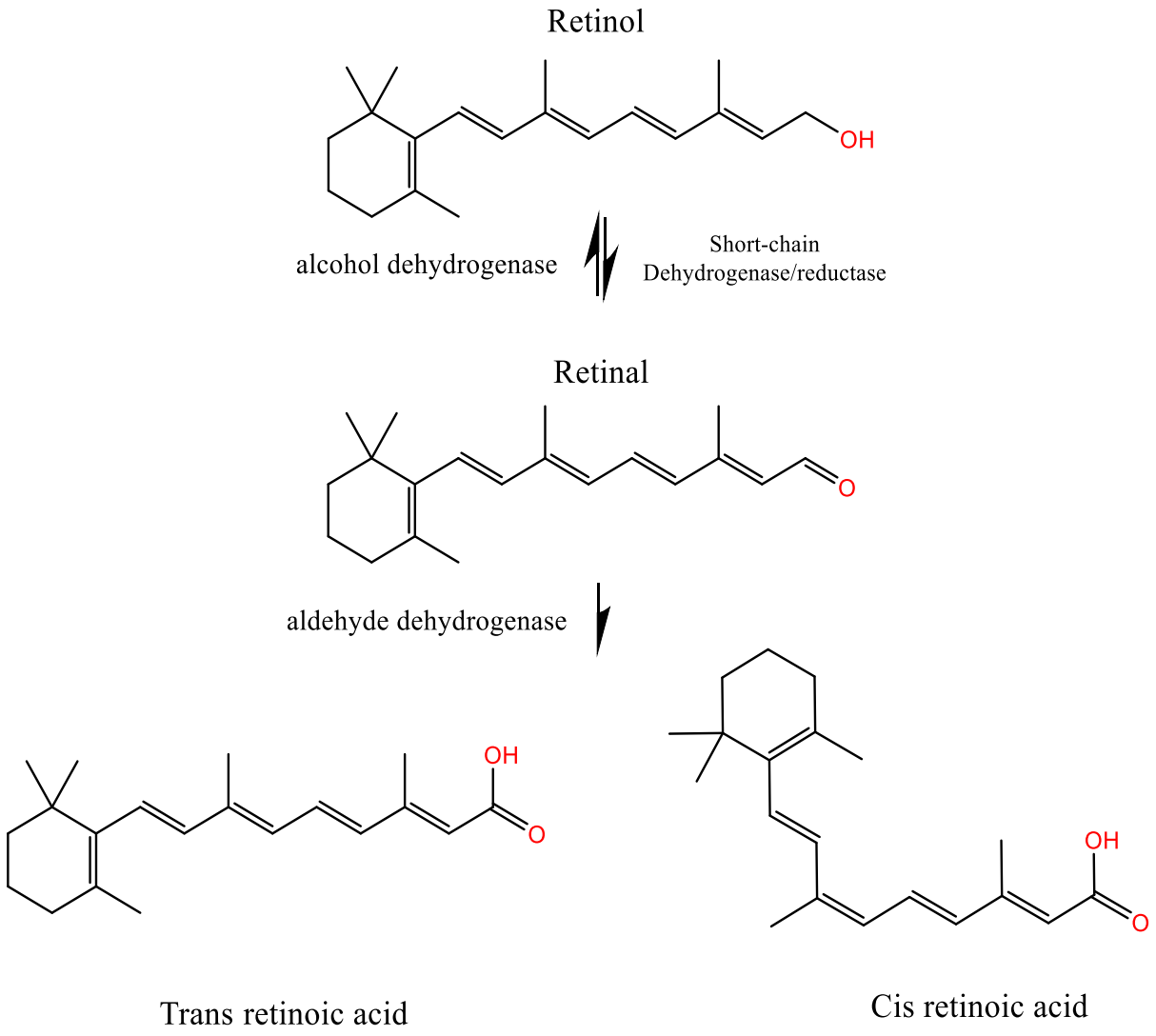


Figure 3. Scheme represents transformation of retinol into bioactive retinoic acid. **[70]**

1.1.2. Vitamin D

Vitamin D is a secosteroid compound stored in adipocytes, it have a broken ring in its structure mainly synthesized endogenously after sunlight (UV-B) exposure or imported via food to calcitriol or eracalcitriol (active forms). It is found in two forms named vitamin D2 (food form) and vitamin D3 (hormonal active form) (figure 4) and plays an important role in human health, it helps:

- Intestinal Ca^{++} absorption (calcium metabolism) which prevents increased risk of osteoporosis **[73]**.
- Diabetes by regulating glucose levels **[74]**.
- Hypertension by inhibiting the renin-angiotensin-aldosterone system (RAAS) which is a hormone within the body controls fluid and electrolyte balance by combined effects on the heart and blood pressure so it is responsible for the regulation of blood pressure and cardiovascular disorders **[75]**.
- Immune diseases by regulating gene expression of most important target genes for immune regulation like hepcidin antimicrobial protein (HAMP), cathelicidin (CAMP) and β-defensin 2 (DEFB4), since the calcitriol receptor called the vitamin D3 receptor (VDR) is expressed by a variety of immune cells, including dendritic cells, monocytes, neutrophils, and T cells **[76]**.

In humans Vitamin D2 and D3 are not active it will be converted firstly in the liver in form of 25-hydroxyergocalciferol and 25-hydroxycholecalciferol respectively, then in the kidney in form of 1,25-dihydroxyergocalciferol and 1,25-dihydroxycholecalciferol respectively. The mentioned vitamin D metabolites will activate the vitamin D receptor in the body. This has a direct effect on the genome and the expression of more than 1000 human genes **[77]**.

The vitamin D known foods sources are fatty fish, cheese, eggs, and milk or direct supplementation **[78]**.

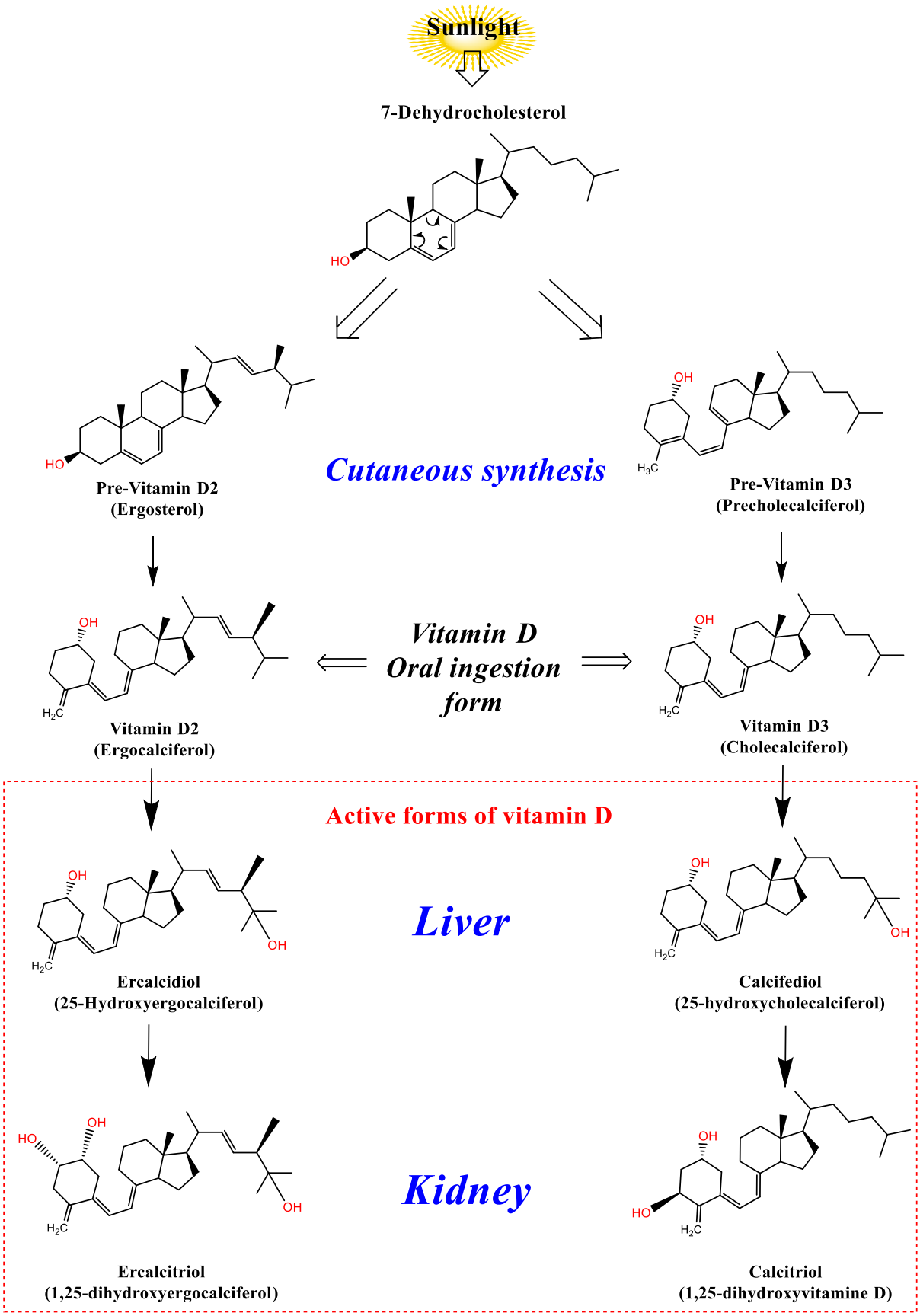
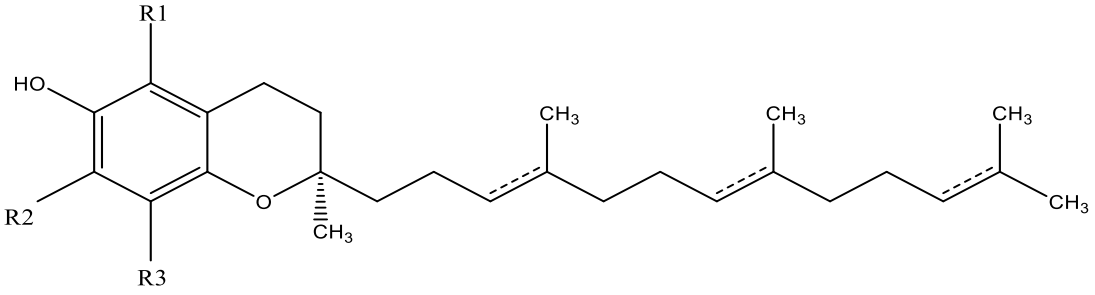


Figure 4. Scheme represents transformation of 7-Dehydrocholesterol into bioactive Eracalcitriol and Calcitriol.

1.1.3. Vitamin E

Vitamin E is a family of fat-soluble micronutrients have antioxidant activity that can protect the oxidation of polyunsaturated in the membranes **[79]**, it mainly composed of four tocopherols formed by a chromanol ring with a phytyl carbonyl chain and four tocotrienols with a chromanol ring and an unsaturated tail, the vitamin E family is divided to four major classes named α, β, γ, and δ, (figure 5) the proportions depends highly on the number and position of methyl groups on the chromanol ring and the food source, α -tocopherol reported as the active form in humans due to involvement of α -tocopherol transfer protein (α -TTP) since it shows high preferential binding affinity to it. It helps regulate the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and modulate signal transduction **[80]**. The main source is the vegetable oils, nuts, corn, and soybean **[80]**.



	Compound	R1	R2	R3	Phytyl chain
Tocopherols	α	CH_3	CH_3	CH_3	Saturated
	β	CH_3	H	CH_3	Saturated
	γ	H	CH_3	CH_3	Saturated
	δ	H	H	CH_3	Saturated
Tocotrienols	α	CH_3	CH_3	CH_3	Unsaturated
	β	CH_3	H	CH_3	Unsaturated
	γ	H	CH_3	CH_3	Saturated
	δ	H	H	CH_3	Unsaturated

Figure 5. Chemical 2D structures of the vitamin E family **[80]**.

1.1.4. Vitamin K

Vitamin k represents the naphthoquinone derivatives compounds such as phylloquinone (vitamin K1), menaquinone (vitamin K2), and menadione (vitamin K3) (figure 6). It is responsible for maintaining bone mass, vascular calcifications, and have important role in blood coagulation factors, after absorption; the vitamin K is transported by chylomicrons lipoprotein through the lymphatic circulation to the liver and other tissues **[81]**. Vitamin K1 is synthesized by plants and present the active form in the human diet **[82]**. Vitamin K2 is formed by menaquinone units differ by the number of isoprenoid side chains (n) it is named menaquinone vitamin K or MK-n and include 13 type (MK-1 to MK-13), MK-4 was reported the most active in human body. Vitamin K2 is a derivative from bacteria and fermented products **[83]**. While vitamin K3 is a synthetic product represents the menadione unit and generally used for animal feed **[84]**. The main source is green leafy vegetables, vegetable oils, and margarines **[81]**.

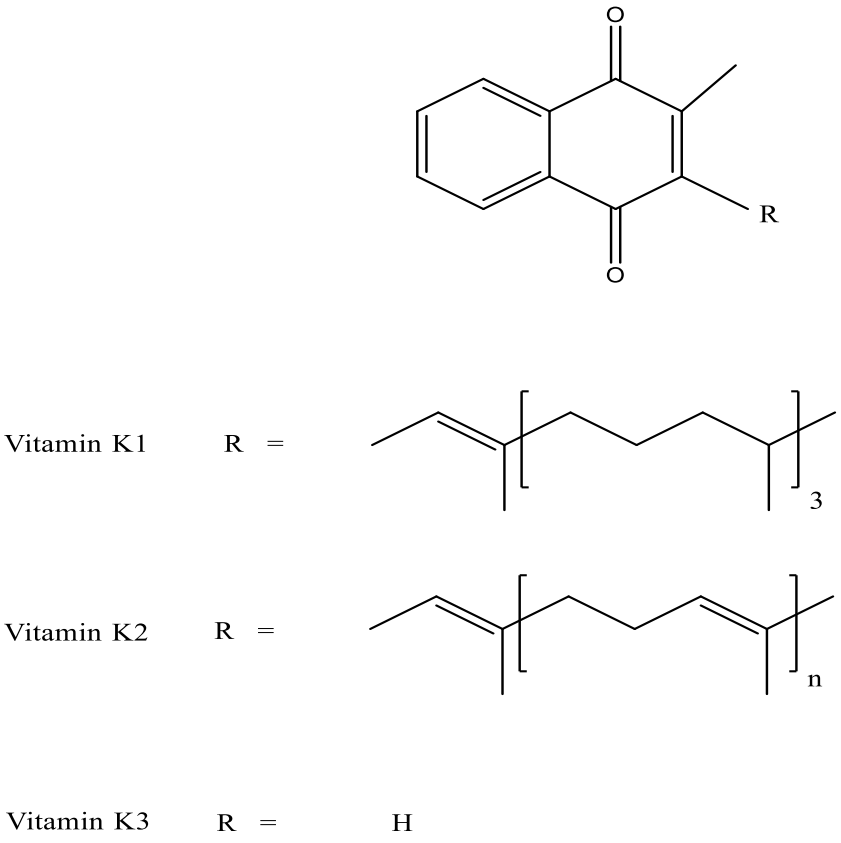


Figure 6. Chemical 2D structure of vitamin K family **[85]**.

1.2.Water-soluble vitamins

Water-soluble vitamins are substances that dissolve in water and cannot be stored in the body, their molecular weight generally < 450 g/mol and mostly secreted in the urine, it usually comes as cofactors form then converted to vitamins form via digestive enzymes like nucleosidases, peptidases, and phosphorylases in the intestinal lumen and intestinal cells like nicotinic acid (NAD) in hydrogen carrier, or prostetic form like pantothenic acid (CoA) in acyl group transferring (fatty acid metabolism), it cannot be synthesized by humans (with the exception of niacin) and must be obtained from food like meat, poultry, and red fish or dietary supplement, their stores may last for days to weeks only, so they must be taken daily **[86]**.

1.3. Vitamin B family

Thiamin, niacin, riboflavin, pantothenic acid, pyridoxine, folic acid, cyanocobalamin, and biotin are the vitamin-B family (table 2). **Thiamin** (vitamin B1) is an essential component for maintaining normal body homeostasis, it converts glucose to energy which is essential for the muscles and nerves, it plays a fundamental role in reducing cellular oxidative stress, precursor to coenzyme thiamin diphosphate (ThDP) **[87]**, energy production, normal cellular functions, growth, and development. Humans cannot synthesize thiamin **[88]**.

Riboflavin (vitamin B2), reported to have crucial role in DNA and protein synthesis, which support growth, cell maintenance, muscle development, apoptosis, inflammatory process, and hair coat **[89]**. Riboflavin enters in the production of other B complex vitamins; it is an electron carriers in redox reactions in diverse metabolic pathways via its two cofactors flavin adenine dinucleotide (FAD) presented in high amounts in foods and flavin mononucleotide (FMN) presented in small amounts, it is also known as antioxidant in the regeneration pathway of glutathione (free radical scavenger) **[90]**.

Niacin (vitamin B3), or nicotinic acid is an important vitamin synthesized by plants and animals from tryptophan amino acid, it is responsible for boosting HDL cholesterol levels and

lower LDL cholesterol in certain treating doses. Mainly is a precursor of the two coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) which are crucial cofactors in hydrogen transfer in the metabolism pathway of macronutrient **[91]**.

Pantothenic acid (vitamin B5) is an essential nutrient used by the body to synthesis acyl carrier protein and coenzyme A (CoA) **[92]**. CoA is essential for acetyl and acyl groups transfer, fatty acid synthesis and degradation. It helps generates energy from carbohydrates, fats, and proteins **[93]**.

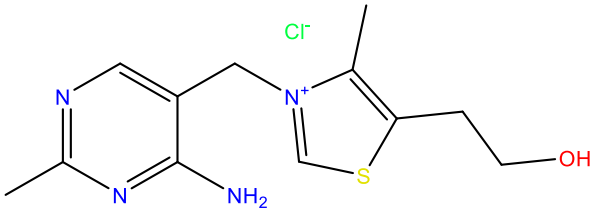
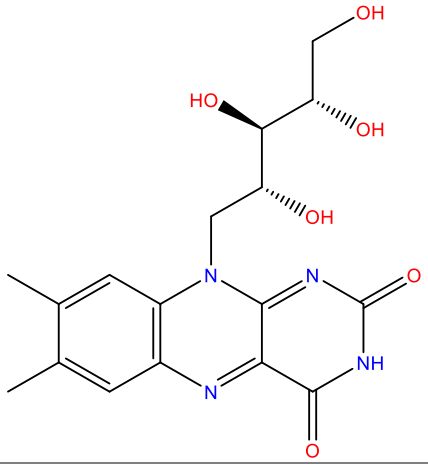
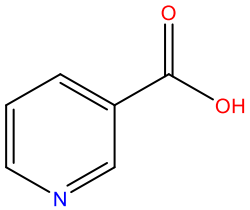
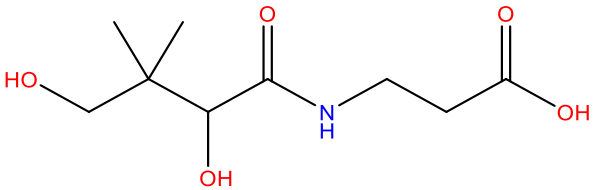
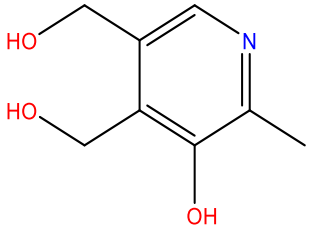
Pyridoxine (vitamin B6) is a group of related six compounds like pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their respective 5′-phosphates (PLP, PNP, and PMP) used by the body as a coenzyme in the metabolism of glycogen, sphingoid bases and more than 100 enzymes involved in the amino acids metabolism including decarboxylases, racemases, aminotransferases, and dehydratases these enzymes depends highly on the PLP cofactor. PLP and PMP are abundant forms in animal tissues; while PN and PNP are plant-derived compounds, sometimes comes in the form of a glucoside **[93]**.

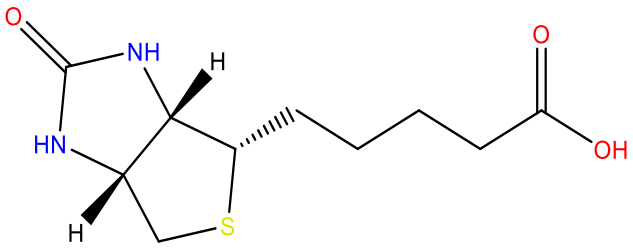
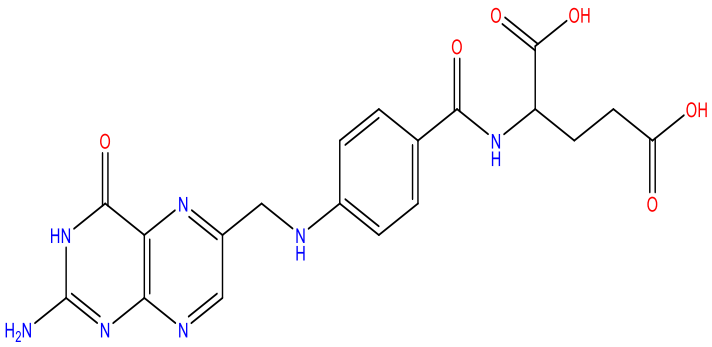
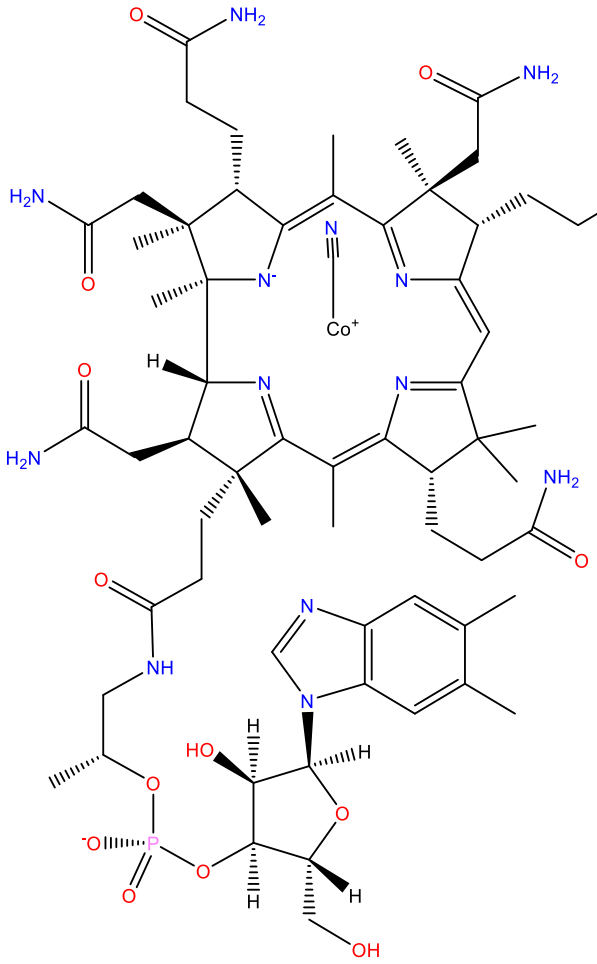
Biotin (vitamin B7) or vitamin H is a group comprises eight forms derived from plants, bacteria, algae, and yeast **[94]**, only the biotin-D form occurs naturally with its complete vitamin activity, it is an essential coenzyme involved in the gluconeogenesis, fatty acid, and amino acid synthesis that includes carboxylases enzymes like: 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase, acetyl-CoA carboxylases propionyl-CoA carboxylase **[93]**.

Folic acid (vitamin B9 or folate) and **cyanocobalamin** (vitamin B12) are two closely related B complex vitamins, they have multiple roles in the body including DNA and RNA metabolism (synthesis of dTMP from dUMP) mainly in the prevention of uracil incorporation into DNA when folic acid is absent, uracil accumulates and incorporates into DNA instead of thymine, these can leads to mutations **[95]**, they have role in red blood cells production which prevent

anemia and hyperhomocysteinemia (high homocysteine), in the immune system responses of lymphocytes and natural killer cells activity **[96]**, vitamin B9 reported to have antiviral properties to the 2019-novel Coronavirus Main Protease (2019- nCoV Mpro) **[97]**.

Table 2. Vitamin B family chemical 2D structures, major functions, and their RDAs.

Vitamin B family	Chemical structure	Major role
Thiamine (vitamin B_1) **RDA :** 1.20 mg		Reducing cellular oxidative stress **[87]**
Riboflavin (vitamin B_2) **RDA :** 1.30 mg		DNA and protein synthesis **[89, 90]**
Niacin (vitamin B_3) **RDA :** 16.00 mg		Precursor of NAD and $NADP^+$ in hydrogen transfer **[98]**
Pantothenic acid (vitamin B_5) **RDA:** 5.00 mg		Fatty acid Metabolism **[99]**
Pyridoxine (vitamin B_6) **RDA:** 1.70 mg		Amino acids metabolism **[93]**

Biotin (vitamin B_7) **RDA:** 0.03 mg		Macronutrients metabolism **[93]**
Folic acid (vitamin B_9) **RDA:** 0.40 mg		Erythropoiesis (Red blood cells production) **[100]**
Cobalamin (vitamin B12) **RDA:** 2.40 µg		Cofactor for methionine synthase and l-methylmalonyl-CoA (prevents folate accumulate) **[93]**

RDA: recommended daily amount

Vitamin B4 (adenine), **Vitamin B8** (inositol), **Vitamin B10** (para-amino benzoic acid – PABA), and **Vitamin B11** (salicylic acid) are no longer labeled vitamins, as they no longer fit the official definition of a vitamin; essential and required for normal human growth and are required to be obtained by diet because they cannot be manufactured by the human body. However, many are still in use and recommended for a variety of health needs as other nutritional supplements **[101]**.

1.4. Vitamin C

Vitamin C, also known as ascorbic acid or ascorbate is an essential micronutrient reported to be the most important vitamin in life (figure 7), its dietary intake higher than most vitamins due to the low storage capacity of the body **[102]**; it has metabolic uses in all living organisms, it is known by its potent antioxidant activities related to its ability to donate electrons which protect from oxidative stress, it can be a cofactor for gene regulatory enzymes and collagen structure stabilizing enzymes like di/monooxygenase (enzymes that incorporate one hydroxyl group into substrates) **[103]** and many metabolic enzymes, it contributes in the innate (non-specific) and adaptive immune system **[104]** by enhancing the differentiation and proliferation of B- and T-cells, prevents and treat respiratory infections, supports epithelial barrier function against pathogens **[105]**.

It has anti-microbial activities by generating the reactive oxygen species humans are unable to synthesize vitamin C because of their deficiency in L-gulono-gamma-lactone oxidase enzyme, which is responsible for catalyzing the terminal step in its biosynthesis **[106]** the recommended daily intakes for vitamin C in healthy people are 100 mg to 200 mg/day **[107]**, it should be sufficient for potential disease risks. The vitamin C can be found in foods include guavas, bell peppers, kiwifruit, strawberries, oranges, papayas, broccoli, tomatoes, kale, and snow peas **[108]**.

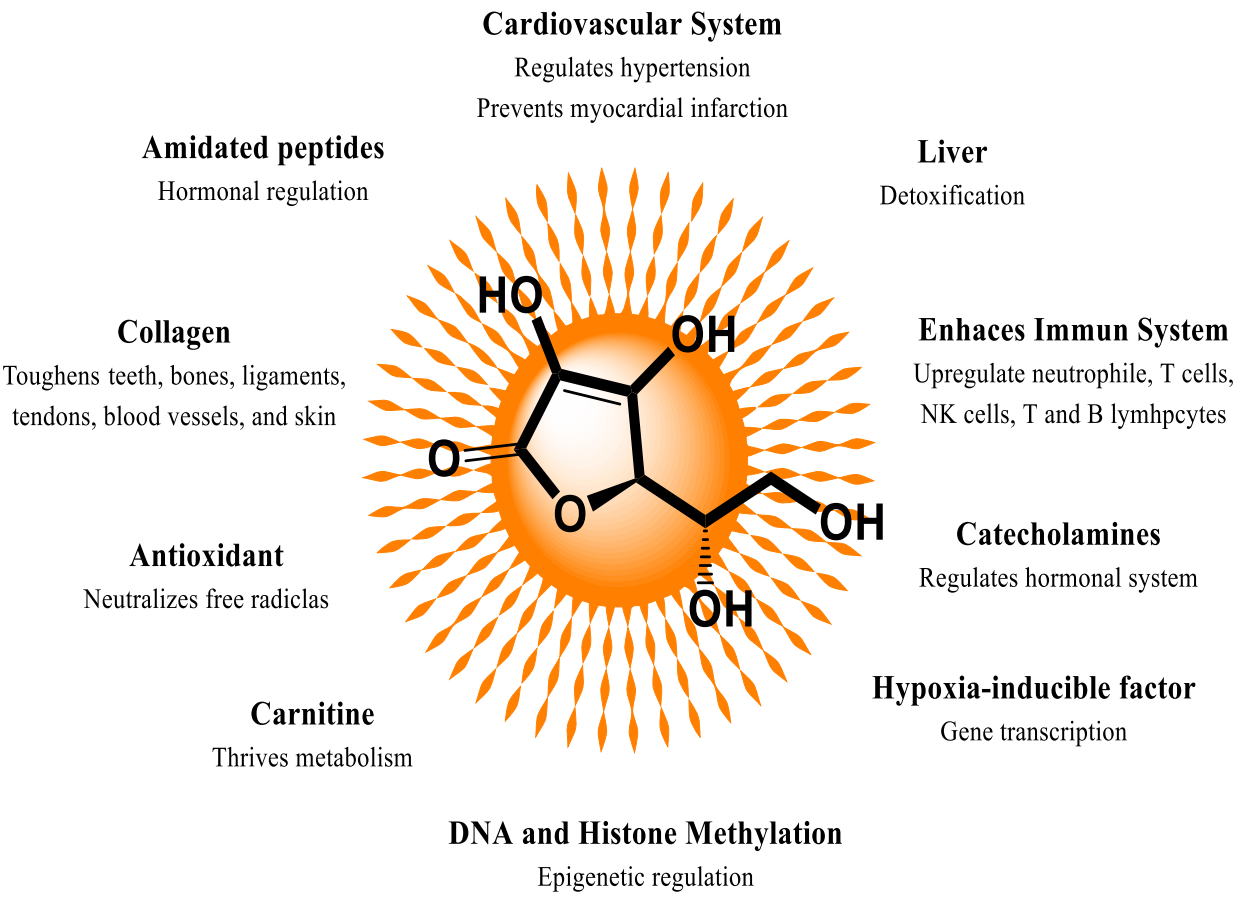


Figure 7. Chart represents numerous functions of vitamin C in the body collected from different sources **[109-111]**.

2. Vitamin Deficiencies

All studied vitamins in this thesis proved essential for normal physiological functions, cell growth and development with respect to the daily requirements; supplementation programs have made diseases such as scurvy (vitamin C deficiency) **[102]** or pellagra (niacin deficiency) **[112]** rare.

Additionally, deficiency leads to a variety of serious adverse consequences like hemolytic diseases **[113]**, neurological disorders, and metabolic syndromes **[114, 115]**. Excluding vitamin deficiencies linked to pregnant, lactating women, young children and athletics, due to their relatively high requirements for these compounds, however, imbalance intake may also risk of developing certain diseases **[116]**.

2.1. Water-soluble vitamins related symptoms and diseases

Vitamin B1 deficit was linked to neurological syndromes like Wernicke-Korsakoff syndrome caused by alcohol abuse; vitamin B1 based treatment was reported effective **[117]**. It can cause limb weakness, ataxia, including peripheral neuropathy and paraesthesias **[118]**.

Vitamin B2 is highly demands in pregnancy since its crosses the placenta, low intake leads to riboflavin deficient or ariboflavinosis in the infant **[90]**. The vitamin B2 deficiency leads also to cheilitis, angular stomatitis, glossitis, oral ulcers, sore throat, watery itchy bloodshot eyes and seborrheic dermatitis **[90]**. **Vitamin B3** deficiency can lead to pellagra that includes dermatitis, diarrhea, and dementia **[112]**. **Vitamin B5** deficiency is rare due to its abundance in all foods, except in people with severe malnutrition, however, it can cause metabolic perturbation and neurodegeneration in Huntington's disease **[119]**.

Vitamin B6 deficiency is often related to depression and confusion **[120]**, seborrheic dermatitis **[121]**, microcytic anemia (decreased hemoglobin synthesis) **[113]**, and epileptiform convulsions **[122]**, low vitamin B6 levels is often related to depletion in neurotransmitters such as dopamine, serotonin, and γ-aminobutyrate due to its biochemical role as cofactor (PLP) for decarboxylases that are involved in neurotransmitter biosynthesis **[123]**.

Vitamin B9 and **vitamin B12** deficiencies are related, it can lead to neural tube defects in pregnant women, megaloblastic anemia, gastrointestinal problems, fatigue and weakness, memory loss, weight loss, and cancer **[114, 115]**.

Vitamin C deficiency results in impaired immunity and higher susceptibility to infections with weakness, bleeding from gums, and teeth loss, in case of severe deficiency; Scurvy disease is highly expected **[102]**. In turn, infections lower vitamin C levels due to enhanced inflammation and metabolic requirements. In contrast, higher (one-gram) doses of the vitamin C are required to compensate metabolic demand. However, imbalanced intake can lead to

hypo/or hypervitaminosis C, eventually, the dietary intake of vitamin C should be 100–200 mg/day **[102]**.

2.2. Fat-soluble vitamins related symptoms and diseases

Vitamin A deficiency damaged growth and bone formation, adverse reproductive capacity, and diminished immunity **[118]**. Xerophthalmia, pediatric ocular morbidity, and night blindness **[124]**. **Vitamin D** deficiency is now recognized as a pandemic especially in non-existent sun areas, sun exposure remain the major source of vitamin D for humans, other sources are few, the lack of vitamin D was always related to body fatigue and depression issues, it can also causes rickets in children, osteoporosis, fractures in adults, cancers, autoimmune diseases, hypertension, and infectious diseases **[125]**.

Vitamin E deficiency cause neurologic abnormalities, especially ataxia characterized by sensory loss, and retinitis pigmentosa due to free radical mediated neuronal damage **[126]**. **Vitamin K** deficiency is linked to coagulopathy, and hemorrhagic diseases especially in newborns **[127]**, including neonatal bleeding called also “Vitamin K Deficiency Bleeding” or VKDB **[128]**, chronic gastrointestinal disorders **[129]**.

3. Previous works

Vitamin A was first discovered back in 1906 and first synthesized in 1947. Vitamin A activity was always linked to its chemical structure composed of β-ionone ring and isoprenoid chain. In recent years, research interest in vitamin A has increased due to its toxicity and epidemiological observations linking to it, like cancer, childhood morbidity, and mortality from infectious disease. The Ten-State Nutrition Survey in 1968-1970, conducted the first study measurements of serum vitamin A levels in United State of America. Population groups have been performed in the first National Health and Nutrition Examination Survey (NHANES I), 1971-1974, for persons ages 3-74; in the second study by National Health and Nutrition

Examination Survey (NHANES II), 1976-1980, for children ages 3-11; and in the Hispanic Health and Nutrition Examination Survey (HHANES), 1982-1984, for persons ages 4-74 **[130]**.

A meta-analysis conducted 20 controlled trials between 1986 to 1992 published by Glasziou, P. P., and D. E. Mackerras **[131]** aimed to study the effect of vitamin A supplementation on morbidity and mortality from infectious disease in particular respiratory and gastrointestinal disease (Table 3).

Table 3. Vitamin A studies in infectious diseases

Trials No.	Period	No of volun/pati	Affiliation/ Country	Theme research	Age (years)	Control	Studies conclusion	Meta-analysis
12	1986-1992	93 151	International	Role in infectious diseases	05-67	Vitamin A Placebo	Regeneration of mucosal barriers	**[132-142]**
12	1932-1992	18 690	Africa Asia Europe	Measles disease	< 06	Vitamin A Placebo	Reduction in mortality	**[143]**
12	1984-1993	137 674	Africa Asia Brasil	Childhood pneumonia	0-08	Vitamin A Placebo	Reduction in mortality in the 6–11month age only	**[144]**
01	1988-1994	2 592	International	Head, neck, and lung cancer	61 (Mean)	Vitamin A N-acetylcysteine Placebo	No effect	**[145]**

No: number **volun/pati:** volunteer/patient

Vitamin D was first discovered in 1935 by Windaus *et al* **[146]** and the Windaus and Bock identified vitamin D3 in 1937 **[147]**, the first study on vitamin D was achieved in 1952 by Carlsson *et al.*, **[148]** and Bauer *et al*, **[149]**, when they discovered the major role of vitamin D in bones, they show its direct implication in the mobilization of calcium from the bone into the plasma compartment **[149]**.

Another important advance in vitamin D research was done in 1958 by Lamm and Neuman **[150]** in attempt to study bone mineralization, they found that vitamin D was responsible for the high levels of serum calcium and phosphorus, which are the normal process of bone mineralisation, this study was later clarified by Underwood *et al.*,**[151]**. Studies then turned to

an understanding of the metabolism of vitamin D and its role on thyroid gland and organs such as heart, intestine, liver, bone and kidney. Some of the available data in this context were summarized in table 4.

Table 4. Major trials on vitamin D effects in human body.

Trials No.	Period	No of volun/pati.	Affiliation/ Country	Theme research	Age (years)	Control	Studies conclusion	Meta-analysis
11	1987-2008	716	Western Europe	Blood pressure	48-74	Placebo	No effect	**[152]**
05	1960-2009	1237	Europe USA	Bone fragility	60 (Mean)	Vitamin D3, calcium	Prevents falls	**[153]**
23	2002-2014	65 455	Europe Americas Middile east	Obesity	15-80	Vitamin D3	Prevents obesity	**[154]**
51	1981-2010	354	Europe USA Australia India	Heart disease	31-89	Vitamin D3 Calcium carbonate Placebo	No effect	**[155]**
01	1991-1992	130	USA	Bone mineral density	51.9 (Mean)	vitamin D3 Calcium carbonate	Increase bone mineral density	**[156]**

No: number **volun/pati:** volunteer/patient

Vitamin E was first discovered in 1922 by the American scientists Herbert McLean Evans and Katherine Scott Bishop **[157]**, later, the Swiss Nobel laureate synthesized the vitamin E in 1938 **[158]**, the first study on vitamin E was achieved in 1936 by Schoorl P *et al.*, **[159]**, the aim of the study was to determine the role of vitamin E in the sterility of female rat.

Studies conducted between the 1950s and 1960s focused more on defining the role of vitamin E:

- In poultry nutrition, including its role in growth and development **[160]**.
- Vitamin E deficiency in monkeys (effect on creatinine excretion) **[161]**.
- Vitamin E deficiency in young rats (Abnormalities of the eye occurring) **[162]**.

Few studies reported its role in human body until 1960, studies turned to the vitamin E role in the human body especially its role in lipid metabolism in man **[163]**.

By the year 1970, studies on its role in cancer, in infant nutrition, its antioxidant activity was well developed. Some of the available data in this context were summarized in table 5.

Table 5. Major trials on vitamin E role in human body.

Trials No.	Period	No of volun/pati.	Affiliation/ Country	Theme research	Age (years)	Control	Studies conclusion	Meta-analysis
19	1996-2004	135 000	Europe Americas Asia Australia	High-dosage (≥400 IU/d) vitamin E	40-84	Placebo Vitamin E	Increase mortality	**[164]**
09	1996-2008	118 765	Europe USA	Haemorrhagic stroke	40-75	Placebo Vitamin E	Increase the risk of haemorrhagic stroke	**[165]**
08	1966-2005	6 642	Europe USA China	Risk of Parkinson's disease	65-75	Vitamin E	No effect	**[166]**
16	1994-2008	137 137	Europe USA Canada	Myocardial infarction	40-80	Vitamin E	Reduces myocardial infarction	**[167]**
05	2006-2011	401	Europe Americas Asia	Fatty liver disease	08-62	Placebo Vitamin E	Improve liver function	**[168]**
12	1993-2005	304 923	Europe Americas Asia	Prostate cancer	18-55	Placebo Vitamin E	Prevents prostate cancer	**[169]**

No: number **volun/pati:** volunteer/patient

Vitamin K was first discovered in 1929 by Henrik Dam, in order to study the effect of a low cholesterol diet in chickens, the chickens tended to develop hemorrhages and start bleeding. Later, Dam confirms that cholesterol was not responsible for hemorrhages. In 1936, Henrik Dam and Edward Adelbert Doisy elucidated the chemical structure, nature, and the role of vitamin K in prothrombin (Factor II), and both received the Nobel Prize for medicine in 1943 for their findings **[170]**.

Studies pursuit on vitamin K in numerous fields like its role in bacteria, in human newborn infant, in human hyperbilirubinemia, in photosynthesis, fracture healing, and analgesic property. Thirty years later exact function of Vitamin K in the body was discovered **[171, 172]**. Some of the available data in this context were summarized in table 6.

Table 6. Major trails on vitamin K role in human body.

Trials No.	Period	No of volun/pati.	Affiliation/ Country	Theme research	Age (years)	Control	Studies conclusion	Meta-analysis
21	1985-2004	983	International	Treatment of Excessive Anticoagulation	18-73	Placebo Vitamin K	No effect	**[173]**
13	1998-2005	258	Japan	Prevention of Fractures	15-78	Vitamin K1-K2	Prevents fractures	**[174]**
27	2004-2017	12 888	International	Vascular diseases (Vascular stiffness (VS) and vascular calcification (VC))	69 (Mean)	Placebo Vitamin K	Reduce VC, but not VS	**[175]**
16	1980-2010	2 978	Europe Japan Canada USA	Lumbar and femoral neck Bone mineral density (BMD)	25-68	Vitamin K1-K2	Increased BMD at the lumbar spine but not at the femoral neck	**[176]**
21	2004-2018	222 592	International	Cardiovascular diseases (coronary heart disease)	21-77	Vitamin K1-K2	Reduce the risk of coronary heart disease	**[177]**
06	1951-1993	21 493	International	Role in pregnancy (neonatal bleeding)	22-40	Placebo Vitamin K	No effect	**[178]**

No: number **volun/pati:** volunteer/patient

Vitamin B complex were discovered in 1889, by Christiaan Eijkman, in its trials on beriberi disease **[179]**, an endemic condition that caused weakness, weight loss, confusion, and sometimes death. In 1906, English biochemist Frederick Gowland Hopkins suggested a connection between nutrition and beriberi, suggesting adding “accessory food factors”. Along with the macronutrients (fats, proteins and carbohydrates) **[180]**. In 1911, Casimir Funk, a Polish biochemist proposed the word “vitamin” **[181]**. Later, chemists throughout the world raced to isolate, characterize and synthesize vitamins. In 1930s, Merck and Robert Williams had isolate and produce thiamine (vitamin B1) **[182]**.

Experiments on vitamin B begun in 1936 upon animals and insects to understand the basic nutritional role **[183]**, content in foods, and the influence of environmental conditions.

Through the late 1960s, B-vitamin deficiency was the subject of scientists worldwide, after 1980 studies pursuit on vitamin B in numerous fields like; its role in cancer and anemia. Some of the important data in this context were summarized in table 7.

Table 7. Major trails on vitamin B function in human body.

Trials No.	Period	No of volun/pati.	Affiliation/ Country	Theme research	Age (years)	Control	Studies conclusion	Meta-analysis
19	1988-2017	16 754	Asia Europe America Australia	Esophageal cancer	30-79	Placebo Vitamin B	Decrease the risk of Esophageal cancer	**[184]**
27	1950-2004	43 754	Asia Europe America Australia	Colorectal cancer	63 (Mean)	Placebo Vitamin B9	Prevents colorectal cancer	**[185]**
14	1996-2011	54 913	Europe USA Canada Australia	Cerebrovascular disease (homocysteine Levels)	52-68	Vitamin B12,B9, B6	Reduce homocysteine levels	**[186]**
10	1996-2016	12 268	International	Breast cancer	23-80	Vitamin B2	No effect	**[187]**
31	1998-2018	17 029	International	Cognitive function (Mental state)	61-80	Placebo Vitamin B12,B9, B6	No effect	**[188]**
09	2008-2013	1 104	Europe America Australia	Anemia after sleeve gastrectomy	33-48	Placebo Vitamin B12	Prevents anemia	**[189]**

No: number **volun/pati:** volunteer/patient

Vitamin C was first discovered in 1928, by Albert Szent-Györgyi in its trials on adrenal glands, he isolated a substance called 'hexuronic acid' **[190]**. Later, in 1939, Charles Glen King confirms that 'hexuronic acid' is the vitamin C **[191]**.

Studies on vitamin C structure starts in 1950s after determined its crucial role in scurvy disease (vitamin C deficiency), over the next ten years (1960-1970) studies conducted its role in diseases like: atherosclerosis, hypercholesterolemia, viral diseases, bones diseases, and metabolism of drugs. In the 1980s and the following 1990s, vitamin C had witnessed a revolution in all the fields of medicine; in cancer, immunity, asthmatic diseases, and metabolism **[111]**. Some of the important data in this context were summarized in the table 8.

Table 8. Major trials on vitamin C function in human body.

Trials No.	Period	No of volun/pati.	Affiliation/ Country	Theme research	Age (years)	Control	Studies conclusion	Meta-analysis
10	1997-2019	1 671	Asia Europe USA	Patients with sepsis	58 (Mean)	Vitamin C Hydro-cortisone	No effect	**[192]**
29	1994-2010	1 407	International	Blood pressure (systolic or diastolic)	22-74	Placebo Vitamin C	Reduce both systolic or diastolic	**[193]**
10	1993-2013	17 696	USA Sweden China Australia	Breast cancer	65 (Mean)	Placebo Vitamin C	Reduce the risk of mortality	**[194]**
18	1993-2011	217 454	Asia Europe USA	Stroke disease	40 (Mean)	Vitamin C	Reduce the risk of stroke disease	**[195]**
52	1998-2013	1 324	International	Endothelial function	18-84	Placebo Vitamin C	Improved Endothelial function	**[196]**
13	1996-2010	556	Europe Mexico Japan	Serum uric acid levels	19-81	Placebo Vitamin C	Reduce serum uric acid	**[197]**

No: number **volun/pati:** volunteer/patient

III. Studied medicinal plants and fungus

In our study, we selected two plants such as: *Peganum harmala* and *Cupressus sempervirens* with the fungus *Inonotus hispidus*, based on their potential in secondary metabolites: phenolic compounds and alkaloids. The botanical classifications, geographical distributions, and the related maps were obtained on August 25, 2021 from Global Biodiversity Information Facility: https://www.gbif.org/).

1. *Peganum harmala* L.

Common local name: Harmel**, الحرمل.**

1.1. Taxonomy of the plant

Kingdom :	*Plantae*
Phylum	*Tracheophyta*
Class :	*Magnoliopsida*
Order :	*Sapindales*
Family :	*Tetradiclidaceae*
Genus :	*Peganum* L.
Species :	*Peganum harmala* L.

1.2. Botanical Description

Peganum harmala is an herbaceous perennial, with straight, twiggy stems, up to 80 cm; leaves deeply divided into strips; white star-shaped flowers, with 5 linear sepals and 5 elliptical petals; numerous stamens (up to 15) with yellow anther, elongated, more or less globose capsule-shaped fruits, with 3 chambers, containing several dark brown seeds **[198]** (figure 8).

Flower

Fruit and leaves

Seeds

Figure 8. Photos represent different parts of Harmel: *Peganum harmala* L (Obtained on August 25, 2021 from USDA Plants Database) **[199]**.

1.3. Geographical distribution

Peganum harmala (African rue) known also by the local name "Harmel" **[200]**, it is a perennial plant native of the arid zones of the north of Africa, the Mediterranean Sea, the Middle East, Turkey, Pakistan, and India. It is introduced and naturalized in regions of the southwestern of USA, North Europe, some areas of South Africa and Australia **[198]**. The worldwide distribution of this plant is shown in figure 9.

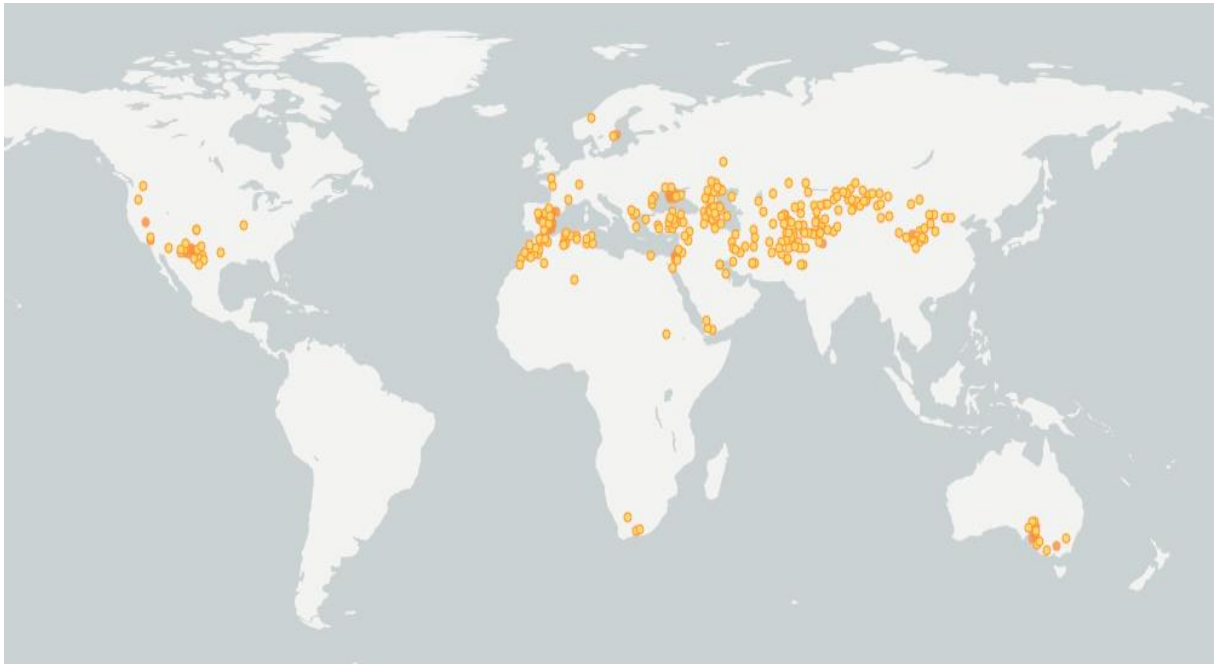


Figure 9. Map of the world distribution of *Peganum harmala* L **[201]**.

1.4. Properties and medicinal uses

Peganum harmala reported to have numerous pharmacological activities for the relief of pain and as an antiseptic agent. It have antibacterial, antifungal, antiviral, antioxidant, antidiabetic, antitumor, antileishmanial, insecticidal and cytotoxic activities **[202]**. The seeds of harmel have also been used as galactagogue, emmenagogue and dewormer, in decoction and ointment, it is used for the treatment of fevers and in frictions to treat rheumatism and in some dermatological diseases **[198]**, it is contraindicated during pregnancy, due to its abortive and mutagenic activities **[203]**. Harmaline, and harmine as two alkaloids were reported to have multiple activities like antioxidants **[204]**, antifungal **[205]**, antiviral **[206]**, anti-parasites **[207]**, and antitumor and neurotoxic effects **[208]**. Harmaline and harmine inhibition effect to bovine milk xanthine oxidase has not yet been studied.

1.5. Previous work

Peganum harmala represents an important source of secondary metabolites like alkaloids moreover; essential oils, its active molecules proven effective in numerous biological activities

and until today, the research of new biological activities ongoing. The table below shows some previous works indexed in four known databases; Nature, Science direct, Springer link, and PubMed, the search field in the title, abstracts, and keywords was "*Peganum harmala*" as the basic term.

Table 9. Major works devoted to the study of *Peganum harmala* activity.

Database	Publication No.	Year	Activities studied	Used terms
Science Direct	89	1997-2021	Antitumor Antiviral Antiseptic Anticancer Antifungal Enzyme inhibitors	*-Peganum harmala, -Essential oils, -Alkaloids,*
PubMed	158	1953-2021		
Springer link	61	1950-2021		
Nature	29	1950-2021		

2. *Inonotus hispidus* (Bull.) P.Karst.

Common local name: Sorret Elbtoum, **سرة البطم.**

2.1. Taxonomy

Kingdom :	*Fungi*
Phylum :	*Basidiomycota*
Class :	*Agaricomycetes*
Order :	*Hymenochaetales*
Family :	*Hymenochaetaceae*
Genus :	*Inonotus* P.Karst.
Species :	*Inonotus hispidus* (Bull.) P.Karst.

2.2.Botanical Description

The basidiomycetes are called the cap fungi. This group includes the agaricales or lamellar fungi, most of which are edible and poisonous. However, it also includes microscopic organisms such as yeasts or plant parasites such as rusts. It is characterized by the presence of reproductive cells, the basidia, which produce external basidiospores almost often 4 in number, however, with very varied forms.

A parasitic fungus lives preferably on a variety of trees with caduceus leaves like *Fraxinus*, *Quercus*, *Sorbus*, *Malus* and *Pistacia*, it produces an important quantity of yellow-brown pigments. The **Cap** can reach 30 cm in diameter, initially rounded, orange, and fuzzy, like a squashed peach, but grows larger and thinner (and darker) with age, take the form of a rounded plate, the interior flesh is pale brown, it can be very large at maturity **[209]**.

Gills: there are no gills, the spores are released from pores on the underside of the cap, the pore surface is initially cream-colored, then darkens and then to brown with age. **Stem**: there is no stem; the mushroom grows directly from the side of the tree **[209]**. Tubes rather long, from 2 to 3 cm, with pores blackening with the age. Fibrous flesh, yellow-brown. It is visible all the year on trunks of ash, fruit trees or other hardwoods (figure 10) **[198]**.

Figure 10. Photo represents Sorret Elbtoum, *Inonotus hispidus* (Bull.) P.Karst (Obtained on August 25, 2021 from MYCOBANK Database) **[210]**.

2.3. Geographical distribution

Inonotus hispidus, it is a fungus mainly found in Europe, and the southeastern of USA it can be also found in north Africa (Algeria) and some areas in the northeastern region of Asia and Australia **[198]**. The worldwide distribution map of this fungus is shown in figure 11.

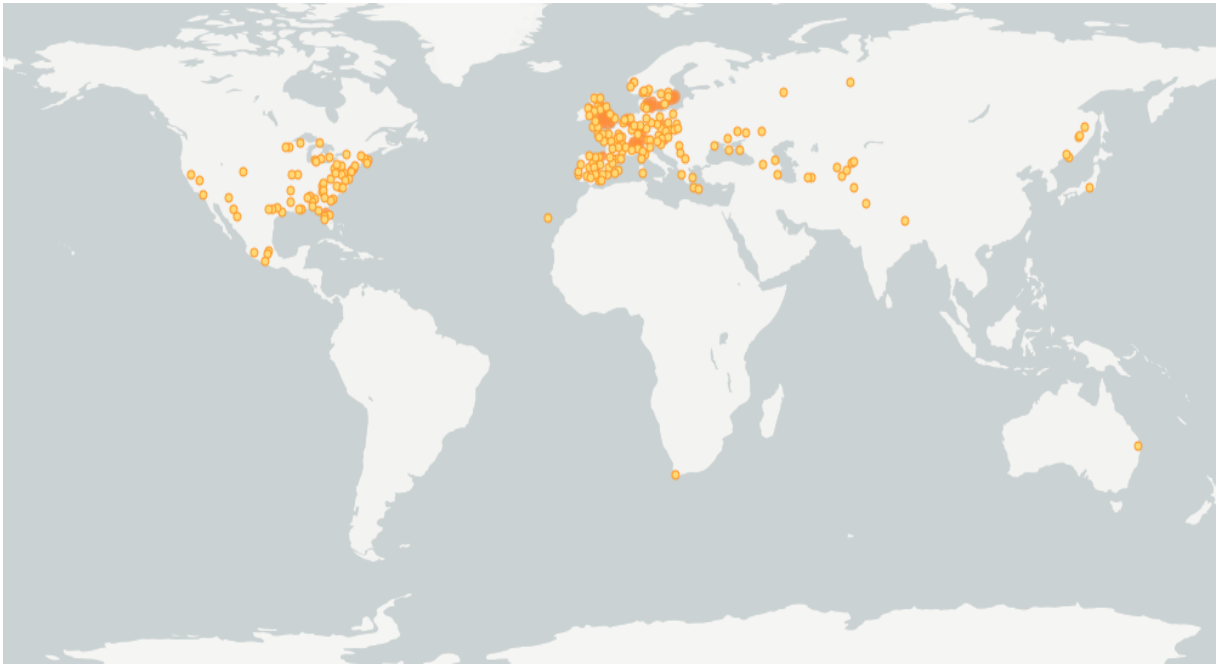


Figure 11. Map of the world distribution of *Inonotus hispidus* (Bull.) P.Karst **[211]**.

2.4. Properties and medicinal uses

Inonotus hispidus has been used as traditional medicine for the treatment of ulcers, inflammations, cancer, and diabetes **[212]**; it can be used as an astringent, anti-microbial, diuretic, and antioxidant.

2.5. Previous work

It was recognized by its richness in phenolic compounds like hispidin and hispolon, studies reported their function in many diseases, other secondary metabolites still little discovered to date, and the search for new biological activities is currently being carried out. The table below shows some previous works indexed in three known databases: Science direct, Directory of Open Access Journals (DOAJ), and PubMed, the search field in the title, abstracts and keywords was "*Inonotus hispidus*" as the basic term.

Table 10. Major works devoted to the study of *Inonotus hispidus* activity.

Database	Publication No.	Year	Studied activities	Used terms
Science Direct	13	1993-2021	Function in human immune cells Antiviral Antibacterial Antioxidant Enzyme Inhibitors	- *Inonotus hispidus,* *-Flavonoids*
PubMed	27	1996-2021		
DOAJ	05	2011-2021		

3. *Cupressus sempervirens* L.

Common local name: Saro, السرو.

3.1. Taxonomy

Kingdom : *Plantae*

Phylum : *Tracheophyta*

Class : *Pinopsida*

Order : *Pinales*

Family : *Cupressaceae*

Genus : *Cupressus* L.

Species : *Cupressus sempervirens* L.

3.2. Botanical Description

Cupressus sempervirens are a group of trees with exposed ovules on scales forming lignified cones (pinecones) on the twigs. The seeds are found on the upper surface of the female cones scales. The leaves are generally persistent and narrow, forming needles in some species (pine, fir, spruce), the flowers hold dorsal glands, and the cones are glossy, brown to gray, pendulous, globose to elliptic, 2-3 cm long. Its height can reach 21 m with 0.6 m width, and can last for 150 years **[213]**.

Cones ***Flowers*** ***Seeds***

Figure 12. Photo represents different parts of Cypress, *Cupressus sempervirens* L (Obtained on August 25, 2021 from Global Biodiversity Information Facility: https://www.gbif.org/).

3.3. Geographical distribution

It was native to the Mediterranean basin. However, the plant was distributed in North Africa (Algeria) mainly found in Europe, and the southern region of USA. It can be also found in some areas in the southern region of Asia (India and China), South America, and Australia. It grows in woodlands, interior valleys, and coastal mountains at elevations from 500 m to 2000 m in areas with Mediterranean climate, dry, hot summers and winter rain, or semi-arid habitats, disturbed in areas along roadsides, gardens, parks and cemeteries **[214]**. The worldwide distribution map of this plant is shown in the figure below.

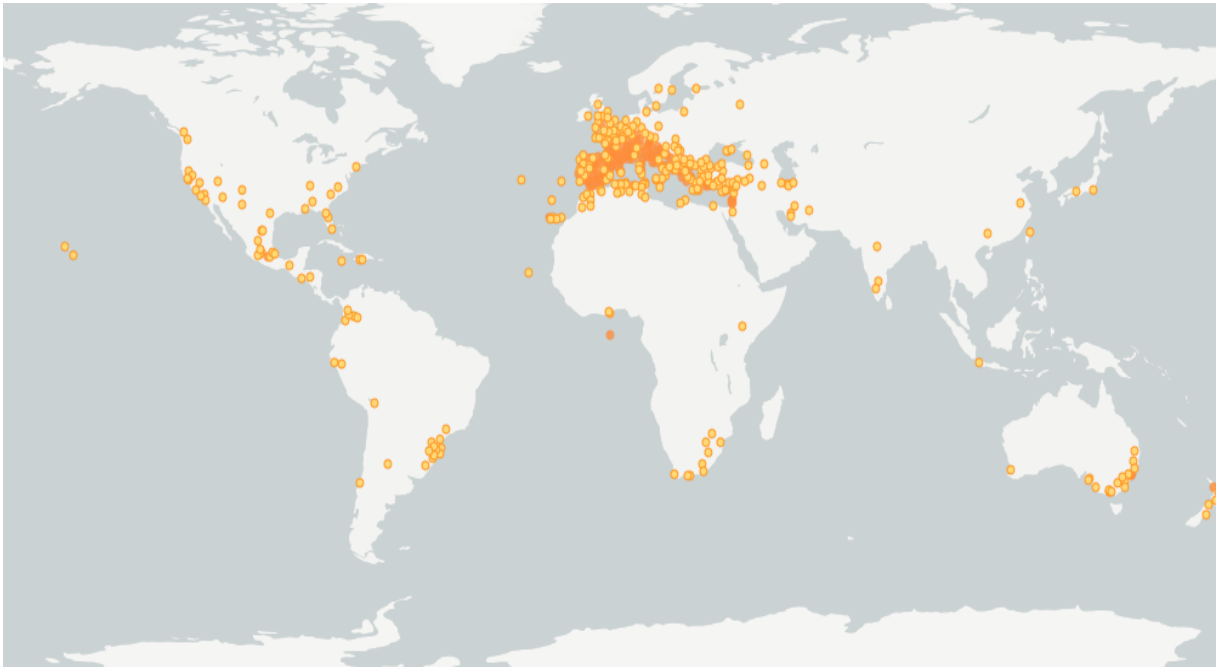


Figure 13. Map of the world distribution of *Cupressus sempervirens* L **[215]**.

3.4. Properties and medicinal uses

Cupressus sempervirens leaves and cones were used for head colds, coughs and bronchitis; cones were used internally as an astringent. Externally, it can be used to treat hemorrhoids, varicose veins and venous circulation disorders. *Cupressus sempervirens* essential oil was used as antiseptic, antispasmodic, anti-stubborn coughs, deodorant, and diuretic (promote venous circulation to the kidneys) **[216]**.

3.5. Previous works

It was recognized by its richness in phenolic compounds, alkaloids, and essential oils like Lignan rhamnoside, Amentoflavone and Isocryptomerin. Studies reported their function as a source of antioxidant, antibacterial and antileishmanial, the search for new biological activities is currently underway. Table 11 shows some previous works indexed in three known databases: Science direct, Directory of Open Access Journals (DOAJ), and PubMed, the search field in the title, abstracts, and keywords was "*Cupressus sempervirens*" as the basic term.

Table 11. Major works devoted to the study of *Cupressus sempervirens* activity.

Database	Publication No.	Year	Activities studied	Used terms
Science Direct	38	1980-2021	Antiviral Antibacterial antifungal Antioxidant Enzyme inhibitors	- *Cupressus sempervirens*, -*Flavonoids*, -*Essential oils* -*Alkaloids*
PubMed	159	1965-2021		
DOAJ	30	2002-2021		

Experimental

In vitro study

Our work was carried out at the Blood Transfusion Center (CWTS) of the public hospital EPH-AHMIDA BENADJILA and the Laboratory of Fundamental Sciences at the University of Amar Telidji-Laghouat.

I. Extraction

1. Plant material

Peganum harmala (*P. harmala*) and *Inonotus hispidus* (*I. hispidus*) were collected in June 2011 in Laghouat City, South of Algeria. The plant *P. harmala* was identified by Pr. Yousfi Mohamed. Pr. Bernard Duhem, Museum « National d'Histoire Naturelle, Laboratoire de Cryptogamie, 12 Buffon Street, 75231 Paris, France», identified *I. hispidus*. All other requirements for plants/fungus collection and storage were respected **[198]**.

Cupressus sempervirens plant was collected from el Dayah region, in June 2019 by Pr. Yousfi Mohamed who also made the sample collection and taxonomic identification at the Fundamental sciences laboratory, Amar Telidji University, Laghouat, Algeria. The plant has been checked with http://www.theplantlist.org.

2. Milk material

The bovine milk used for the enzyme inhibition assays was obtained from a farm in El M'reigha-Laghouat. After being freshly drawn, no filtration, separation, or thermic treatment was performed; the milk was stored at 04 °C then used in the same day for the extraction.

3. Organics and chemicals

The Allopurinol (control), Vitamins E, D3, B9, and C were bought from the pharmacy. Uricase and peroxidase enzymes were purchased from BIOLABO (Les Hautes Rives, Maizy, France). Xanthine substrate, K_2HPO_4 (Dibasic potassium phosphate), KH_2PO_4 (Monobasic potassium phosphate), KCL (Potassium chloride), DMSO (dimethyl sulfoxide), EDTA

(Ethylene diamine tetraacetic acid), reagents were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH Taufkirchen, Germany). All reagents were analytical grades unless otherwise stated.

4. Equipment and procedures

Freezer from SUPERARTIC FIOCCHETTI ECT-F -30 °C (Panagulis, Luzzara, Italy), used for enzymes storing, refrigerated centrifuge from HETTICH ROTANTA 460R (Bäch SZ, Suisse), allows the centrifugation of 2 L of milk bag with regulated temperature option (between -20 °C and +40 °C), Microplate reader from URIT UV660 (Guangdong, China). Allows testing 96 assays in 5 seconds, Universal Oven UF30 (Memmert GmbH + Co. KG). BIOSEALER CR 4 electric sealing machine (Ljungberg & Kögel AB) equipment for sealing milk bags (weld and cut the tubing of the bags containing milk), Semi-automatic plasma extractor (BMS), allows to extracts milk phases using a proportional pressure, Magnetic stirrer (Heidolph-MR 3001 K), used in the extraction protocol of the enzyme, Electronic scale (KERN ABS).

5. Plant extraction and the used parts

Peganum harmala and *Inonotus hispidus* isolated molecules were (Harmine, Harmaline) and (Hispidin), respectively and were purified and published in 2015 **[205]** at the Fundamental sciences laboratory, Amar Telidji University, Laghouat, Algeria and stored in opaque glass bottles until their use.

The *Cupressus sempervirens* seeds and globose cones were used for the first time. The dried and powdered fruit (50 g) was divided into equal parts (a mixture of grains and globose cones), 25 g each (figure 14). The first part was used to extract the flavonoids (FE), for that amount of 25 g; we have macerated it with a triple mixture of Ethanol-Methanol-Water (40:50:10) at room temperature for 2 days; the alcoholic fraction was evaporated. The aqueous fraction was subjected to liquid-liquid extraction hexane and acetate ethyl solvents, the obtained extract was filtered and concentrated to dryness under reduced pressure at 40 °C. The other amount of 25 g

was used to extract the alkaloids as cited in **[217]**. For the alkaloid extract (AE), a quantity of 25 g was soaked in 250 ml of HCl (10 %) for 48 h. Then after filtration, the liquid phase was homogenized with 250 ml NH_4OH (25 %), the next step serves to extract alkaloids by adding chloroform to the aqueous phase. Finally, we separated the organic mixture and dried it using a rotary evaporation at 40 °C to have dry crude extract of alkaloids **[217]**. The experimental protocol is summarized in figure 14.

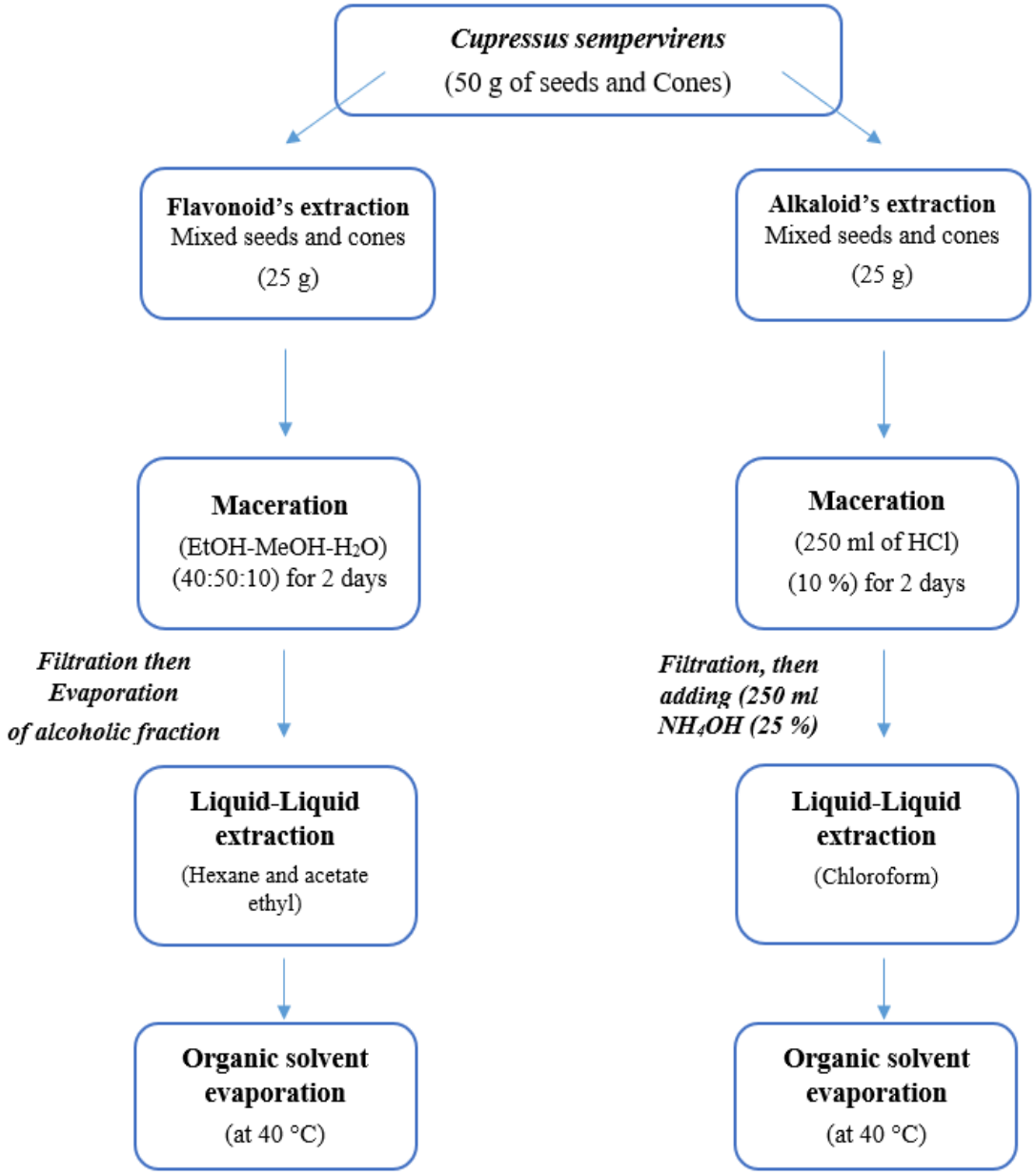


Figure 14. Chart represents the extraction steps of flavonoids and alkaloids from *Cupressus sempervirens* L.

6. Bovine milk separation

The separation process was done according to **[218]** with optimization (figure 15). We used fresh unpasteurized bovine milk (500 ml) from an individual cow, then centrifuged it at 4500 rpm for 35 minutes **[35]**. Afterward, a thick floating white cream was collected and thawed for a moment at 37 °C then dissolved in a double volume of phosphate potassium (K_2HPO_4, 0.2M) containing 1 mM of EDTA **[218]**. Subsequently, the obtained suspension was mixed for 2 hours then centrifuged at 4500 rpm for 20min **[35]**. All the separation steps were strictly done in 4 °C **[219]**. Eventually, the obtained enzyme extracts were centrifuged, and the floats were collected in aliquots of 1.5 ml and conserved at -30 °C for the tests. We decided to study the inhibition using the xanthine oxidase enzyme extract instead of a pure fraction of it as cited in **[219]** to mimic the *in vivo* conditions. All the steps are summarized in the chart shown in figure 15.

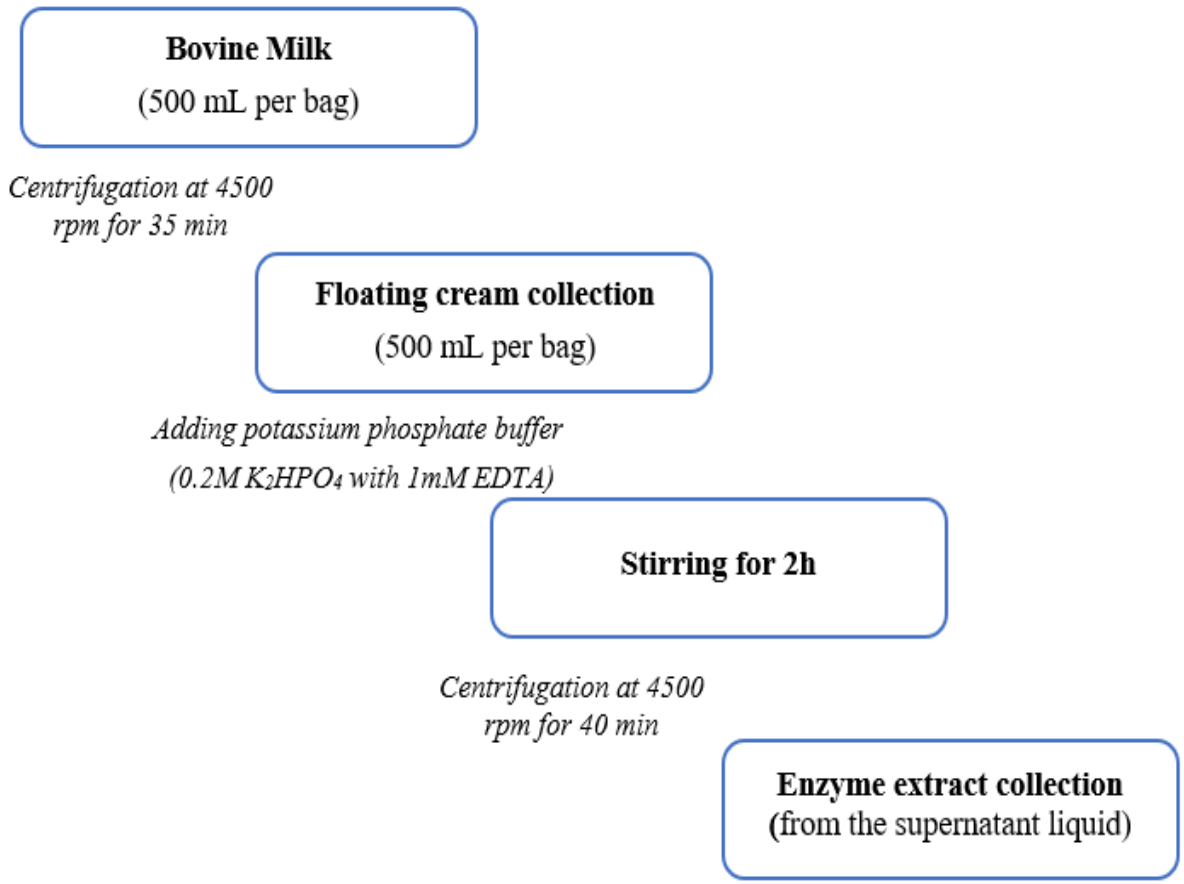


Figure 15. Chart represents the bovine milk xanthine oxidase extraction steps.

II. Enzyme activity

1. Effect of some solvents on the enzyme activity

Before studying the enzyme activity, we tested the effect of some organic solvents frequently used to dissolve hydrophobic molecules (like fat-soluble vitamins) on our enzyme extract. The used solvents were found effective, however, undefined and undetermined percentage can influence the overall enzyme activity, in order to study their effect, we tested:

- Inhibitors dissolution with DMSO, Ethanol, and Methanol solvents.
- Substrate dissolution with sodium hydroxide and sulfuric acid.

In a series of increasing percentages ranging from 10 % to 100 %. Phosphate buffer (PH 7.4) was used as the control solvent. The assay was carried out according to the DED method described by **[35]**, which involves monitoring the rate of appearance of uric acid at 492 nm, for that we incubated BXO extract with the substrate (xanthine) and the reagent using the mentioned solvents.

2. Determination of kinetic constants (K_m and V_{max})

To determine the kinetic behavior of our enzyme, we traced its activity in the absence of the inhibitor depending on the appearance of the product (uric acid), using microplate reader, the initial velocity (Vi) of an enzyme-catalyzed reaction is depending on the substrate concentration [S], for that, we mixed the BXO extract with the xanthine at different concentrations ranging from 65 µM to 390 µM, then the reagent was added as the last component. The activity was measured by taking the absorbance every 20 s for a period of 2440 seconds (40 min) until the end of the reaction, Vi for every xanthine concentration is calculated from the plots of absorbance vs time in its linear part.

The graphics of Michalis Menten (**Vi = f[S]**) were plotted after determining all initial velocities values (Vi) for all obtained xanthine concentrations by determining the rate of product formation using the uric acid absorption coefficient, therefore we have presented the

different parameter using Lineweaver-Burk (**1/Vi = f(1/[S])** and Hanes Woolf (**S/Vi = f([S])** plots, using Microsoft Excel v2016. All the performed assays were done in triplicate.

3. Bovine xanthine oxidase inhibition

The inhibition assay was achieved according to our previous work **[35]** with slight modification, the method used was the double enzyme detection abbreviated DED, it consists of "detecting the uric acid after yielding to quinoneimine with pink-red colored complex" (figure 16) **[35]**. We have modified our previous protocol to economize the reagents.

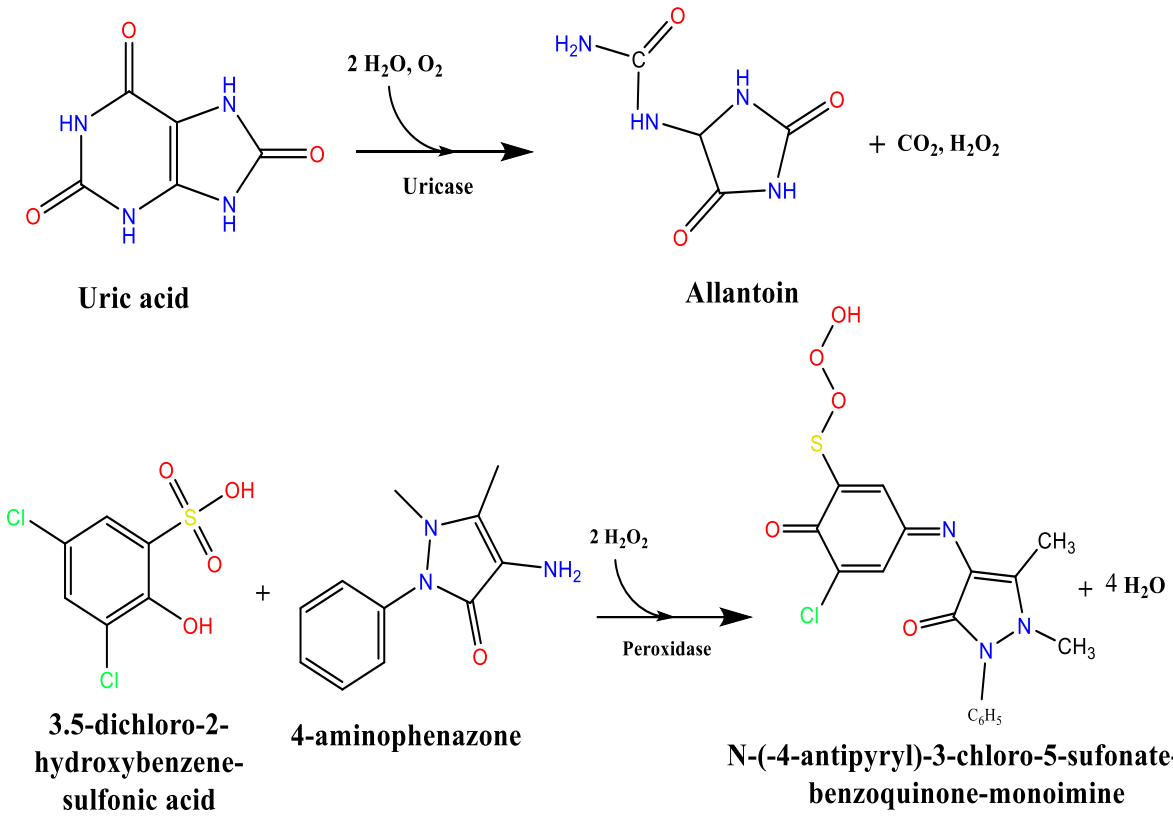
Optimization consists of addressing four elements:

- The maximum absorbance of the control will be at 0.6 in the center of Lambert's law.
- Ensure a volume ratio of 1.2.2 for enzyme, inhibitor and substrate respectively.
- Ensure a maximum volume of reaction medium of 250 µl using small type microplates.
- Ensure to dissolve the substrate in high percentage of phosphate buffer as possible, to avoid the potential inhibition effect of adding strong acids or bases.

The control consists of reacting BXO enzyme extract with the xanthine without inhibitor; it was tested at his maximum activity (equal to double K_m value) with xanthine concentration. The absorbance was read at double wavelength mode in a microplate reader, we used allopurinol as the inhibitor reference to BXO. All experiments were done eight times. All the other condition in the test was respected as cited in **[35]**. The BXO inhibitory activity was expressed as the percent inhibition of BXO (I %) and was calculated as follows:

$$I\ (\%) = \frac{\mathrm{AC} - \mathrm{AE}}{\mathrm{AC}} * 100$$

- **AC** represents the absorbance in the absence of the inhibitor.
- **AE** represents the absorbance in the presence of the inhibitor.



(Fossati 1980 ***[220]****, Bou-Salah 2021* ***[221]****, modified)*

Figure 16. Schema represents the conversion of uric acid into a colored product.

4. Determination of the inhibition constants (Ki)

The enzyme kinetics in the presence of inhibitors was carried out using Dixon (1953) method **[222]**, this latter was chosen as the most accurate among other tested graphical methods for determining the enzyme inhibition type and the dissociation constant (Ki) of our BXO-inhibitors complex **[222]**. The effect on the enzyme velocity is determined at four substrate concentrations (82 (**0.5Km**), 164 (**Km**), 246 (**1.5Km**), and 328 (**2Km**) µM) and over a range of inhibitor concentrations. In a plot of 1/V vs I, we used six concentrations of every inhibitor [I] depending on their IC50 values, the chosen [I] should be between IC25 and IC75, in the presence of four substrate concentrations (0.5 K_m, K_m, 1.5 K_m, and 2 K_m), the enzyme extract was diluted 40 time in the phosphate buffer (pH 7.4), the inhibitors were freshly prepared just prior to performing the experiments with high percentage of phosphate buffer.

The reaction was initiated by adding 22.6 µl of the enzyme extract mixed with 45.2 µl of every inhibitor at various concentrations in micro-wells for 15 min, 45.2 µl of the substrate was added to the mixture, then rapidly 136 µl of the detecting reagent was added, the chosen time interval for measuring the absorbance was confirmed after testing multiple periods (from 20 s to 200 s). Finally, absorbance detection was fixed every 100 s for a period of 11 min at 492 nm, all initial velocities in the presence of the inhibitors was determined from the initial linear portion of absorbance vs time graph using the uric acid absorption coefficient, then linearized using Dixon plot (**1/Vi = f([I])** using Microsoft Excel v2016.

The type of inhibition, Ki (inhibition constant) and V_{max} were determined using the Dixon graph. All the tests were performed in triplicate.

5. Statistical analysis

The one-way analysis of variance (ANOVA) is used in this thesis to determine whether there are statistically significant differences (different from each other) between the means of an independent (unrelated) group.

Data were expressed as mean ± standard deviation (n = 8) in the case of the inhibition test, (n = 3) in the case of the inhibitory kinetics. Data were analyzed by one-way ANOVA using Microsoft Excel v2016, the variable factor was the inhibitors concentration and $p < 0.05$ was considered statistically significant.

In silico study

I. Data collection

1. Structure-activity relationship (SAR)

To understand the main function of BXO and HXO towards its substrate and inhibitors, we used the GOLD v4.0 program for molecular docking. First, the crystal structure of both bovine and human milk xanthine oxidase (PDB IDs: 3nrz and 2ckj) were obtained from the Protein Data Bank (PDB) **[36, 223]** and defined as the receptors (Figure 17). Second, the docking results were treated into the Discovery Studio Visualizer program v20.0 (DSV) by comparing the obtained PLPchem scores (generated by GOLD) and the repetition rate (RR) percentage (generated by DSV) of both allopurinol and hypoxanthine to study the involved amino acids and the formed interactions.

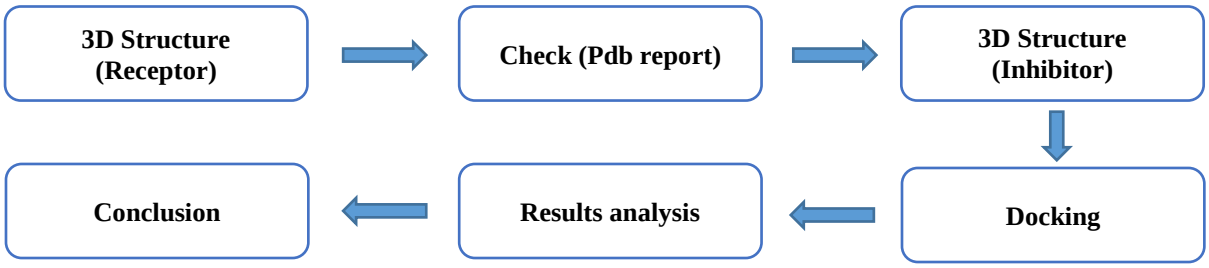
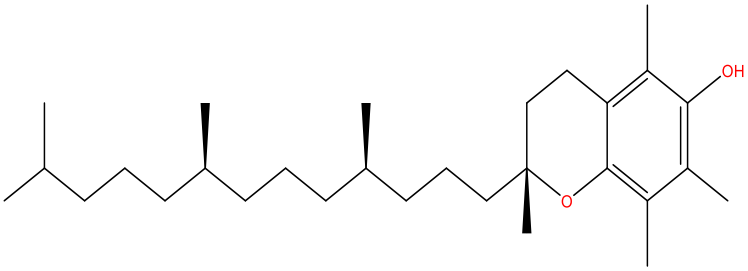
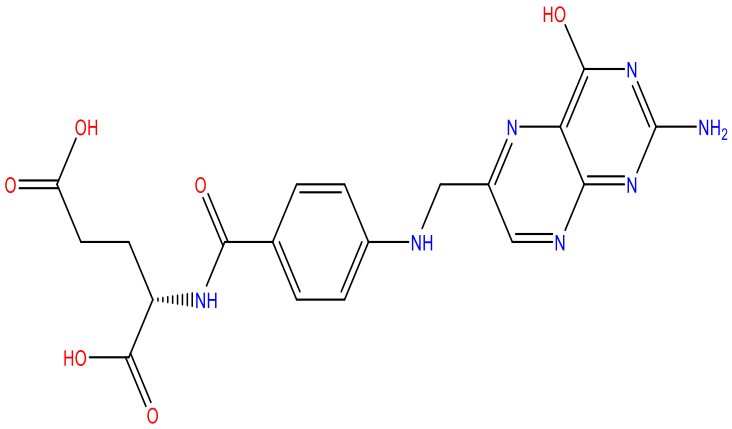
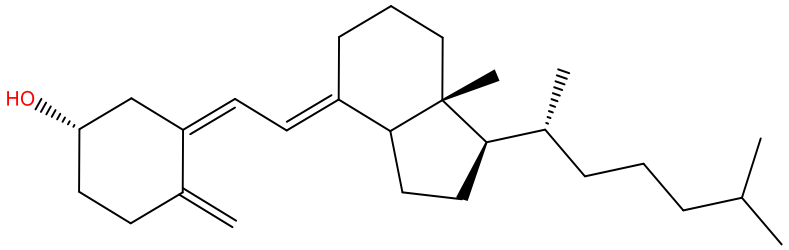
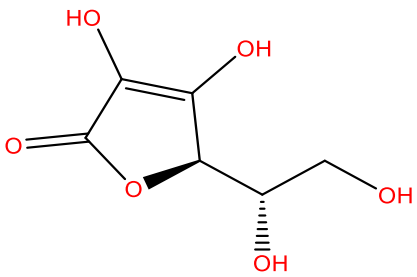


Figure 17. Schema showing the docking procedures.

The used inhibitors were four known vitamins, such as: tocopherol (vitamin E), folic acid (vitamin B9), cholecalciferol (vitamin D3), and ascorbic acid (vitamin C). Natural compounds: hispidin from *Inontus hispidus*, harmine, and harmaline from *Peganum harmala*. From Cypress, 6 purified compounds (five flavonoids and one terpenoid). These ligands were chosen depending on the their solubility, lower weight (< 500 da), availability, and their medical known properties **[224]** (table 12). The inhibitor's two-dimensional structures (2D) of the inhibitors were obtained from PubChem data base in. mol2 format **[225]** and were checked with GOLD program. The topological polar surface area (TPSA) **[226]** values were generated using Chemdraw v16.0.

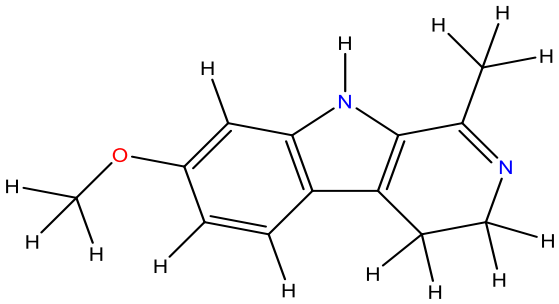
Table 12. The studied compounds 2D structure with their properties.

Inhibitors	Chemical structure	Reference
	Vitamins	
α-tocopherol (vitamin E) BF: $C_{29}H_{50}O_2$ SY: Lipid-soluble RDA: 15.0 mg MW: 433.38 g/mol TPSA: 29.46 Å	 (*R*)-2,5,7,8-tetramethyl-2-((4*R*,8*R*)-4,8,12-trimethyltridecyl)chroman-6-ol	PubChem database **[225]**
Folic acid (vitamin B_9) BF: $C_{19}H_{19}N_7O_6$ SY: Water-soluble RDA: 0.40 mg MW: 441.14 g/mol TPSA: 211.42 Å	 (4-(((2-amino-4-hydroxypteridin-6-yl)methyl)amino)benzoyl)-*L*-glutamic acid	
Cholecalciferol (vitamin D_3) BF: $C_{27}H_{44}O$ SY: Lipid-soluble RDA: 0.02 mg MW: 384.34 g/mol TPSA: 20.23 Å	 (1*S*,*Z*)-3-(2-((1*R*,7a*R*,*E*)-7a-methyl-1-((*R*)-6-methylheptan-2-yl)octahydro-4*H*-inden-4-ylidene)ethylidene)-4-methylenecyclohexan-1-ol	
Ascorbic acid (Vitamin C) BF: C_6H_8O6 SY: Water -soluble RDA: 90 mg MW: 176.03 g/mol TPSA: 107.22 Å	 (*R*)-5-((*S*)-1,2-dihydroxyethyl)-3,4-dihydroxyfuran-2(5*H*)-one	

Purified molecules

Harmaline

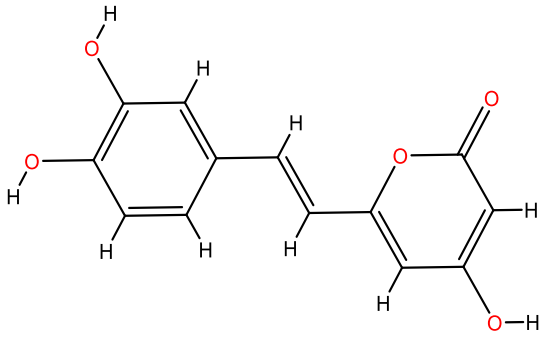
BF: $C_{13}H_{14}N_2O$
SY: Lipid-soluble
MW: 214.11 g/mol
TPSA: 33.62 Å



7-methoxy-1-methyl-4,9-dihydro-3*H*-pyrido[3,4-*b*]indole

Hispidin

BF: $C_{13}H_{10}O_5$
SY: water-soluble
MW: 246.22 g/mol
TPSA: 86.99 Å

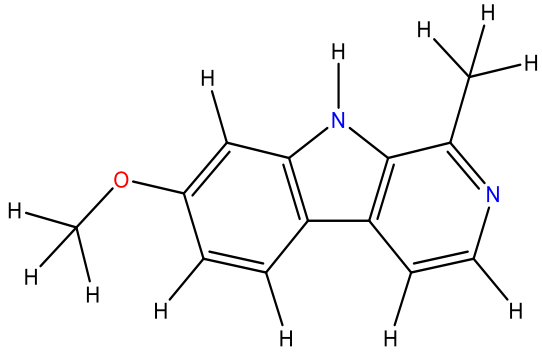


(*E*)-6-(3,4-dihydroxystyryl)-4-hydroxy-2*H*-pyran-2-one

Drug Bank 5.0 **[227-229]** National Academy of Sciences **[230]**

Harmine

BF: $C_{13}H_{12}N_2O$
SY: Lipid-soluble
MW: 212.25 g/mol
TPSA: 33.62 Å

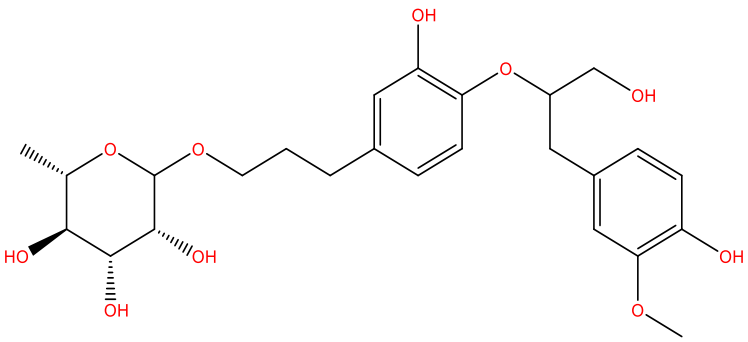


7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole

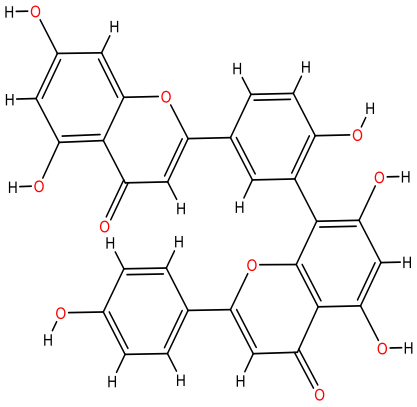
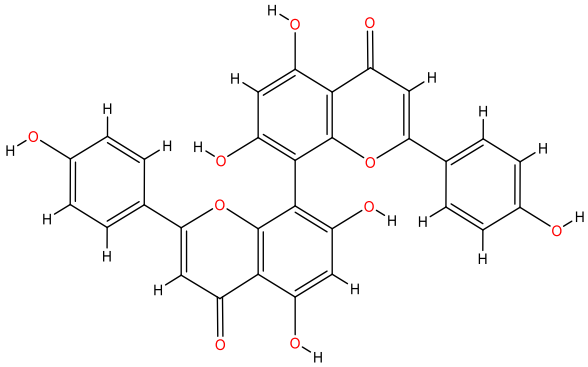
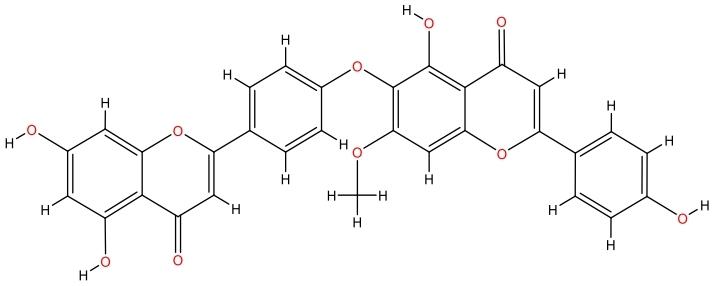
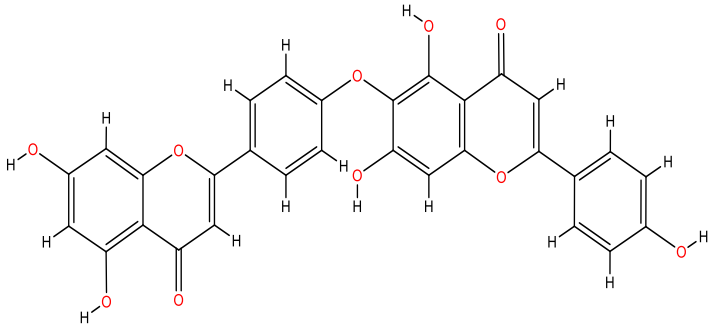
Cupressus sempervirens purified molecules

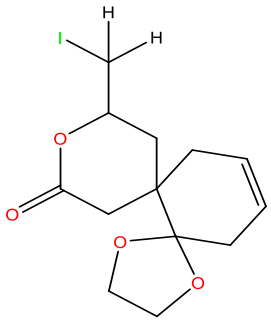
CP1
(glycosylated phenol)

BF: $C_{25}H_{34}O_{10}$
SY: Lipid-soluble
MW: 494.22 g/mol
TPSA: 158.3 Å



(3*R*,4*R*,5*R*,6*S*)-2-(3-(3-hydroxy-4-((1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)propan-2-yl)oxy)phenyl)propoxy)-6-methyltetrahydro-2*H*-pyran-3,4,5-triol

CP2 (biflavonoid) BF: $C_{30}H_{18}O_{10}$ SY: Lipid-soluble MW: 538.46 g/mol TPSA: 173.98 Å	 8-(5-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl) -2-hydroxyphenyl)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one	National Center for Biotechnology Information (NCBI) **[231]**
CP3 (biflavonoid) BF: $C_{30}H_{18}O_{10}$ SY: Lipid-soluble MW: 538.46 g/mol TPSA: 173.98 Å	 5,5',7,7'-tetrahydroxy-2,2'-bis(4-hydroxyphenyl)-4*H*,4'*H*-[8,8'-bichromene]-4,4'-dione	
CP4 (biflavonoid) BF: $C_{31}H_{20}O_{10}$ SY: lipid-soluble MW: 552.11 g/mol TPSA: 151.98 Å	 6-(4-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl)phenoxy) -5-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-4*H*-chromen-4-one	
CP5 (biflavonoid) BF: $C_{30}H_{18}O_{10}$ SY: lipid-soluble MW: 538.46 g/mol TPSA: 162.98 Å	 6-(4-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl) phenoxy)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one	

CP6 (terpenoid) BF: $C_{13}H_{17}IO_4$ SY: Water-soluble MW: 364.02 g/mol TPSA: 44.76 Å		10-(iodomethyl)-1,4,9-trioxadispiro[4.0.5^6.4^5]pentadec-13-en-8-one

TPSA: topological polar surface area **BF**: brute formula **SY**: solubility **RDA**: recommended daily amount

2. Molecular docking settings

The Molecular Docking (MD) was set as a specific docking type given the full flexibility to the inhibitor and catalytic site flexibility of the receptor. The GOLD (Genetic Optimization Ligand for Docking) software **[232]** imposes six steps;

The first step (I); consists of removing the ligands and heteroatoms, and the unnecessary water molecules except the required in the active site; in this case, none of the water molecules were involved in the active site of the receptor.

The second step (II); requires adding hydrogens; 21602 and 9814 hydrogens were added for BXO and HXO respectively, including those necessary to define the correct ionization and tautomeric states.

The third step (III); consists of defining the active site. GOLD proposes multiple ways to define the active site; a list of atoms mode was chosen and defined by nine amino acids such as Glu802, Arg880, Ala910, Phe914, Phe1009, Thr1010, Ala1078, Ala1079, and Glu1261 for BXO. Gln768 Arg881 Arg913 Glu1262 for HXO **[36]**.

The fourth step (IV); the ligands settings, consist of energy minimization using the Hyperchem v8.0 program **[233]**, since GOLD will not modify bond lengths or angles, or rotate rigid bonds **[232]**.

The fifth step (V); selecting a fitness function, GOLD offers a choice of fitness functions: the Piecewise Linear Potential (PLPchem) fitness function was chosen as the best scoring

function in GOLD **[232]**. The used PLPchem score is an empirical fitness function optimized for pose prediction used to model the steric complementarity between protein and ligand **[234]**.

The sixth step (VI); handle the docking solution settings, the number of genetic algorithms (GA) runs set at 100 solutions (results) per inhibitor for the root mean square deviation (RMSD) default settings were used. The best solutions were chosen based on the highest PLPchem score and the ratio of the most repeated poses (RR) in percentage, along with polar hydrogens and hydrophobic interactions formed with the inhibitors inside the active site. The obtained MD results were analyzed using Discovery Studio Visualizer (DSV), v 20.0 **[235]**.

II. Pharmacokinetics properties

1. Oral administration bioavailability (OAB)

The first clinical trial is an important step in drug development to ensure the pharmacokinetics (PK) properties and avoid failure in the last phases. The ADMET prediction tool was applied in this thesis. We used pre-ADMET v2.0 **[236]** server, as the most powerful and cited server in the literature **[237-240]**; it predicts from previous *in vitro* and *in vivo* related assays provided as clinical trials for small molecules, chemical registration, analysis like NMR, MS, HPLC, and others related bio-assay saved in a database.

We verified the OAB of our inhibitors, for that four drug-likeness rules were applied such as CMC-like (comprehensive medicinal chemistry), Lead-like, MDDR-like (MDL Drug Data Report), and Lipinski rule of five **[241, 242]**. This latter allows splitting up drug-like from a non-drug-like structure. According to Lipinski *et al.*, **[241]**, the rule of five matches "with 90% of orally active drugs that have achieved phase II clinical status" **[241]**.

2. Absorption, Distribution, Metabolism, Excretion and Toxicity analysis (ADMET)

The ADMET profile was chosen based on the drug most used parameters; here we describe them:

❖ **Absorption**

- The caco-2 cell permeability (nm/sec) is a human colon epithelial cancer cell line used to predict drug efflux **[243]**.
- Human intestinal absorption (HIA %) is used to track drugs transport targets **[236]**.
- P-glycoprotein inhibition activity (P-gp) was used to investigate drug intestinal penetration **[244]**. It is also a member of the ATP-binding transmembrane glycoprotein family, which can excrete drugs or other exogenous chemicals from cells.
- Water solubility (mg/l) presents the absorption properties.

❖ **Distribution**

- The blood-brain barrier (BBB) penetration is formed by endothelial cells and prevents non-selective molecule to enter the central nervous system; it was calculated as follows (drug concentration in brain/drug concentration in blood) **[236, 245]**.
- Madin-Darby canine kidney (MDCK) cell permeability (nm/sec) is an epithelial cell line of canine kidney origin used as supplementary assessment to the human intestine barrier **[246]**.
- The Plasma protein binding (PPB) percentage is used to estimate the half-life of the xenobiotic distribution from the blood into the tissues where they could be metabolized **[247]**.
- Skin permeability (log Kp, cm/hour) investigates the effects of drug penetration across the skin barrier **[248]** as the distribution property.

❖ **Metabolism**

The cytochromes P450 (CYPs) drug activity were evaluated as the metabolism parameter since CYPs are essential for metabolizing 90% of drugs **[249]**, like cytochrome 2C19, cytochrome 2D6, and cytochrome 3A4.

❖ **Excretion and Toxicity**

The excretion routes are resumed in urine and feces. Finally, the toxicity in the early stages of drug discovery saves both time and developmental costs. Toxicity profile summarizes the:

- Ames tests, which assess the mutagenic potential of drugs towards DNA **[250, 251]**.
- Carcinogenicity prediction in both mice and rats, is the potential of a drug to induce cancer or increase its incidence; it is necessary study for drug-long term treatments.
- Human ether related gene channel (HERG) was reported responsible for modulating the heart function **[252]**.

Results

In vitro study

I. Extraction

1. Enzyme crude and plant material extraction

The average yield obtained was 12.10 % of bovine xanthine oxidase extract (table 13); the enzyme extract activity was checked after reacting with the substrate (xanthine) at 0.05 g/l and the reagent for 30 min. The results show a colored product in purple indicating the presence of uric acid and confirm the enzyme activity according to **[35, 220]**. The extract yield from cypress plant is presented in table 13.

Table 13. Yields from enzyme and extracts.

	Enzyme crude (Mean)	**CEAE**	**CAE**
Yield (%)	12.10	1.20	0.250
Aspect	Milky	Clear yellow	Clear yellow

CEAE: Cypress Ethyl Acetate Extract **CAE:** Cypress Alkaloids Extract

II. Enzyme activity

1. Effect of the solvent on the enzyme activity

The results show that the BXO activity is preserved in the presence of the buffer and ethanol unlike other solvent like: DMSO, methanol, sodium hydroxide, and sulfuric acid, where we observed a decrease in the enzyme activity. The table 14 summarizes the maximum allowed solvent percentage to ensure maximum enzyme activity.

Table 14. Values of maximum allowed solvent percentage.

DMSO	**Ethanol**	**Methanol**	**Sodium hydroxide**	**Sulfuric acid**	**Phosphate Buffer**
25 %	70 %	10 %	< 1 %	< 1 %	**100 %**

2. Determination of the kinetic constants (K_m and V_{max})

The obtained graph (figure 18) shows an increase in the uric acid absorbance associated with the increase in the xanthine concentration, the maximum absorbance recorded in every substrate was between 0.100 and 0.550, the linear portion was defined at 1000s where the formed product concentration is the lowest, and the absorbance vs. time graph is shown in figure 18.

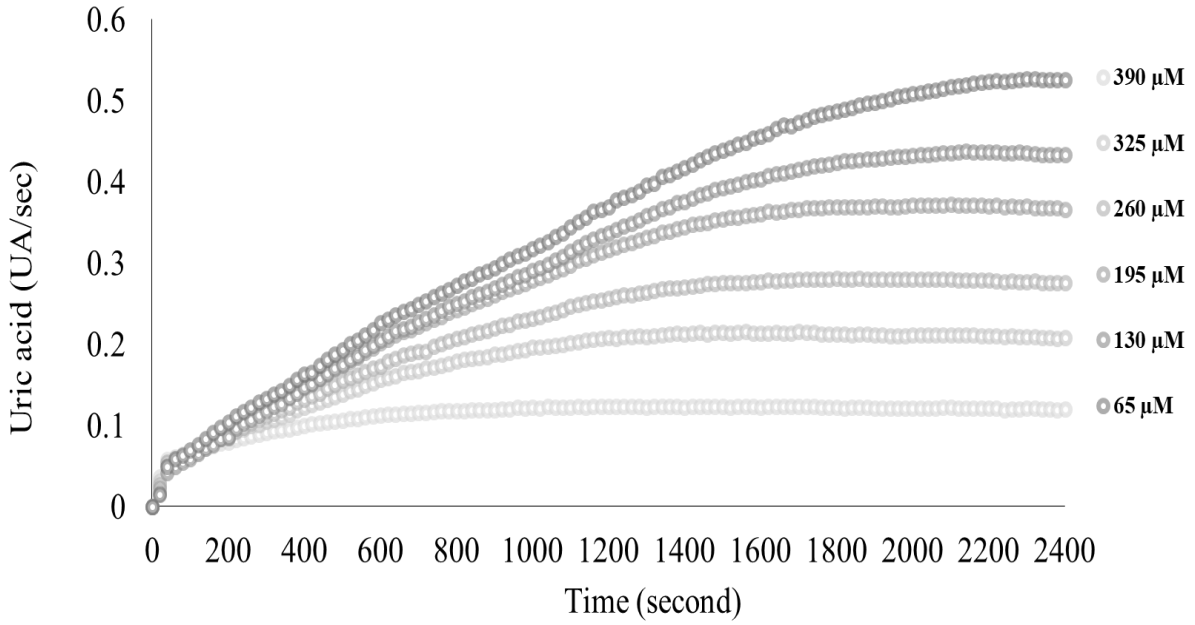


Figure 18. Kinetic of uric acid appearance during 2400 s.

The Vi vs [S] graph, shows that the BXO have a V_{max} value of 37.59 U, and K_m value of 163.55 µM, all the linearized plots are presented in the appendices section.

3. Inhibitory kinetics

The results show significant inhibition to BXO by the used inhibitors; the cypress extracts (ranging from 3 to 9 µg/ml), vitamin B9, vitamin E, Harmine, vitamin C, and hispidin (ranging from 34 to 40 µM) present the most potent inhibition activity with the smallest IC50 value, Harmaline and Vitamin D3 (ranging from 50 to 100 µM) were saved as moderate inhibitors. The best selected inhibitor with the smallest Ki value was Harmine. The change in velocity was recorded as significant to moderate; it ranged from 4 to 37.59 U. All the results are summarized in table 15.

Table 15. Summary of the inhibition assay with kinetic parameters.

Inhibitors	IC50 (µM)	Ki (µM)	V_{max} app (U)	Inhibition type	*P-value*
Vitamins					
Vitamin D3	100.28 ± 0.33	20 ± 0.71	7 ± 0.64	Non competitive Mixed type	
Vitamin E	36.68 ± 1.50	29 ± 1.06	20 ± 0.84	Non competitive Mixed type	
Vitamin C	39.01 ± 0.02	12 ± 1.41	8.3 ± 0.00	Non competitive Mixed type	
Vitamin B9	34.10 ± 0.21	15 ± 1.76	8.3 ± 0.84	Non competitive Mixed type	
Pure molecules					
Hispidin	39.72 ± 0.32	3.5 ± 0.00	37.6 ± 0.93 (V_{max})	Competitive	*Considered statistically significant* *< 0.05*
Harmaline	51.00 ± 1.00	11 ± 2.12	5.55 ± 0.18	Non competitive Mixed type	
Harmine	48.52 ± 1.76	2.5 ± 0.00	4 ± 0.05	Non competitive Mixed type	
Extracts					
CEAE (µg/ml)	8.46 ± 1.98	-	-	-	
CAE (µg/ml)	3.52 ± 0.04	-	-	-	
Control					
Allopurinol	**32.03 ± 0.73**	**6.5 ± 0.35**	**6.66 ± 0.55**	Non competitive Mixed type	

CEAE: Cypress Ethyl Acetate Extract **CAE:** Cypress Alkaloids Extract

4. Statistical analysis

Based on the *p-value* in the ANOVA output, we determined that the differences between the means are statistically significant in all tested cases in this thesis. The p-value was found less than the significance level (0.05); hence, we rejected the null hypothesis and concluded that all the population means are different.

In silico study

I. Molecular docking

According to the best PLPchem score and RR percentage, vitamin E, hispidine, and Cp1 were the best inhibitors compared with the control (allopurinol). The other compounds tested were found to be moderate; Cp6 was the last inhibitor ranked for BXO with the minimum saved criteria. Relatively identical results were found with the HXO model, with the exception of Cp2 and Cp3 being the last ranked inhibitors. Otherwise, the ligands share similar RR values and hydrophobic-like interactions. All tested compounds interacted with catalytic amino acids, with the exception of vitamin D3 (in BXO). In addition, hispidin, harmine, harmaline, vitamin E, and Cp1 retained the greatest number of interactions with catalytic amino acids compared with allopurinol. All results are summarized in table 16.

Table 16. The molecular docking results.

Inhibitors	RR (%)	PLPchem score	Nucleophilic Residues	Interactions type	Length (Å)	Fav/ Unfav bond
Bovine xanthine oxidase						
Hypoxanthine (Substrate)	100	53.86	Arg880	Hydrogen Bond Alkyl T shaped Pi-Pi Stacked Alkyl+Pi Alkyl	1.78 >3.4	11/0
Allopurinol (Control)	100	48.88	Arg880	Hydrogen Bond Alkyl T shaped Pi-Pi Stacked Alkyl+Pi Alkyl	2.09 >3.4	11/0
Vitamins						
α tocopherol (vitamin E)	100	73.27	Thr1010	Hydrogen Bond Alkyl+ Pi Alkyl	2.85 >3.4	20/0
Folic acid (vitamin B_9**)**	100	70.73	Met1038	Hydrogen Bond Pi-Sulfur Pi-Pi Stacked Pi-Pi T-shaped	1.73 >3.4	18/1
Cholecalciferol (vitamin D_3**)**	100	38.12	Ser710	Hydrogen Bond Alkyl	2.95 >3.4	15/2
Ascorbic acid (vitamin C**)**	100	53.75	Arg880	Hydrogen Bond Metal-Acceptor Pi-Sigma	1.97 3.12 2.45	7/1

Purified molecules						
Harmaline	100	52.86	Glu802	Hydrogen Bond Pi-Sigma Amide-Pi+S Alkyl+Pi Alkyl	1.73 >3.4	21/0
Harmine	100	57.08	Glu802	C-Hydrogen Bond Alkyl T shaped Pi-Pi Stacked Alkyl+Pi Alkyl	1.57 >3.4	22/0
Hispidin	100	71.96	Arg880	Hydrogen Bond Alkyl T shaped Pi-Pi Stacked Alkyl+Pi Alkyl	1.98 >3.4	14/0
***Cupressus sempervirens* molecules**						
Cp1	97	**88.09**	Thr1010	Hydrogen Bond Pi-Sigma Amide-Pi-S Alkyl+ Pi Alkyl	1.65 >3.4	19/1
Cp2	72	51.12	His875	C-Hydrogen Bond Pi-Sulfur Pi-Pi Stacked Alkyl+ Pi Alkyl	2.17 >3.4	11/0
Cp3	70	61.08	Lys771	Hydrogen Bond Alkyl T shaped Alkyl+ Pi Alkyl	2.16 >3.4	13/0
Cp4	25	63.35	Ser876	Hydrogen Bond Pi-Sigma T shaped +S Alkyl+ Pi Alkyl	2.50 >3.4	11/4
Cp5	23	63.40	Ser876	Hydrogen Bond Pi-Sigma T shaped +S Alkyl+ Pi Alkyl	2.22 >3.4	13/34
Cp6	0	41.88	Gln1040	Hydrogen Bond Alkyl+ Pi Alkyl	2.42 >3.4	2/0
Human xanthine oxidase						
Hypoxanthine (Substrate)	100	26.08	Met1039	Hydrogen Bond Alkyl T shaped Pi-Pi Stacked Alkyl+Pi Alkyl	2.59 >3.4	8/1
Allopurinol (Control)	100	24.88	Glu1262	Hydrogen Bond Alkyl T shaped Pi-Pi Stacked Alkyl+Pi Alkyl	1.98 >3.4	7/0

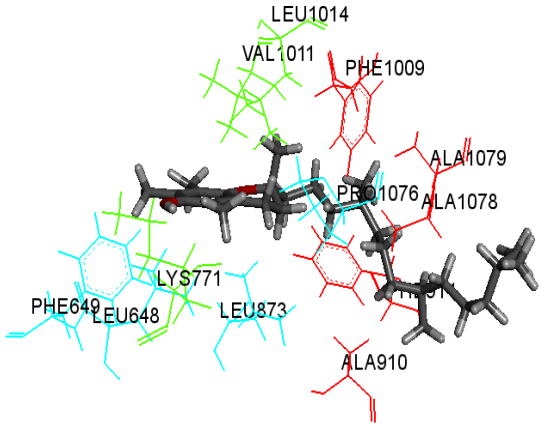
Vitamins						
α tocopherol (vitamin E)	100	65.41	Gln1041	Hydrogen Bond Alkyl+ Pi Alkyl	2.31 >3.4	15/0
Folic acid (vitamin B_9)	100	60.68	Ser1185	Hydrogen Bond Pi Alkyl	1.63 >3.4	11/0
Cholecalciferol (vitamin D_3)	100	68.09	Glu803	Hydrogen Bond Alkyl	1.83 >3.4	8/0
Ascorbic acid (vitamin C)	100	40.48	Ala1079	Hydrogen Bond Pi-Sigma	2.24 2.76	7/1
Purified molecules						
Harmaline	100	48.93	Gln1195	Hydrogen Bond Pi-Pi Stacked Alkyl+Pi Alkyl	2.00 >3.4	11/0
Harmine	100	50.99	Gln1195	C-Hydrogen Bond Pi-Sulfur Pi-Pi Stacked Alkyl+Pi Alkyl	2.21 >3.4	11/0
Hispidin	100	62.04	Glu1262	C-Hydrogen Bond Pi-Sulfur Pi-Pi Stacked Alkyl+Pi Alkyl	2.11 >3.4	12/0
***Cupressus sempervirens* molecules**						
Cp1	96	**72.77**	Ser1083	Hydrogen Bond Alkyl+ Pi Alkyl	1.79 >3.4	14/4
Cp2	0	-1.69	Ile1193	Hydrogen Bond Pi-Sigma Pi Alkyl	1.55 >3.4	20/142
Cp3	0	-9.15	Lys1046	Hydrogen Bond Pi Alkyl	1.91 >3.4	19/143
Cp4	96	31.34	Gln1195	Hydrogen Bond Alkyl+ Pi Alkyl	1.52 >3.4	18/14
Cp5	100	33.17	Arg913	Hydrogen Bond Pi Alkyl	2.02 >3.4	14/6
Cp6	100	41.59	Gly798	Hydrogen Bond Alkyl+ Pi Alkyl	1.73 >3.4	7/0

PLPchem: Piecewise Linear Potential **Fav/ Unfav**: Favorable / Unfavorable **S**: Pi-Pi Stacked **C**: carbon

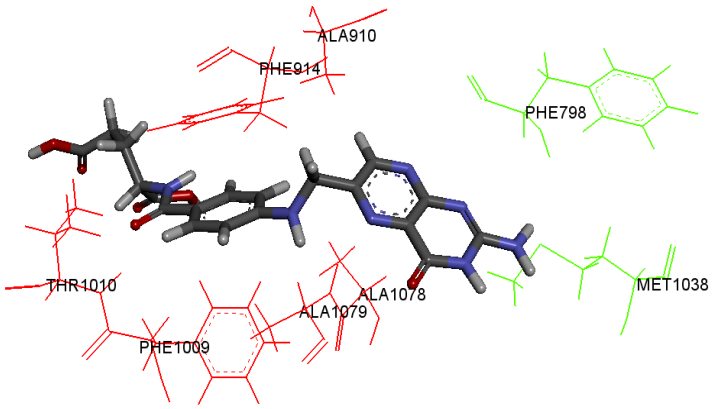
The BXO docking results were presented in 3D demonstration according to the best pose (with the highest PLPchem score) and amino acid types (catalytic, binding or contributing). The obtained results are summarized in the figure 19.

Vitamins

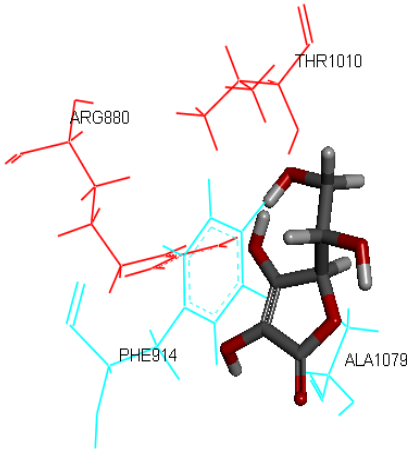
Vitamin E



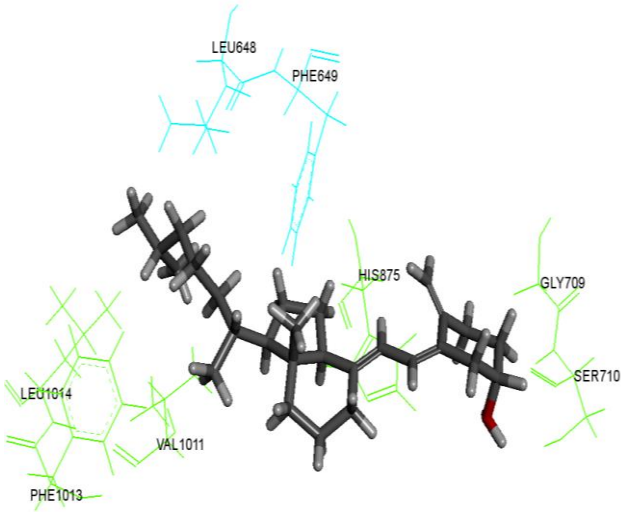
Vitamin B9



Vitamin C

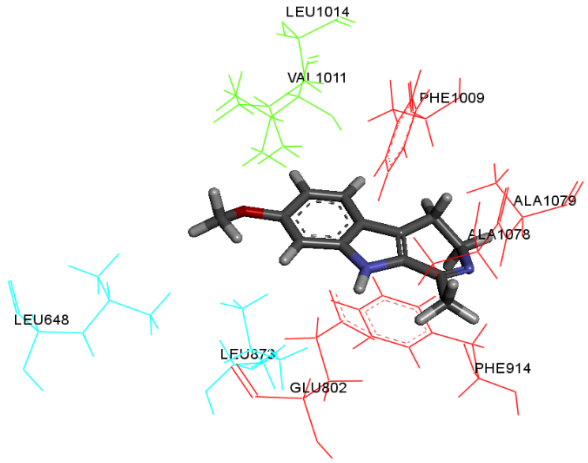


Vitamin D3

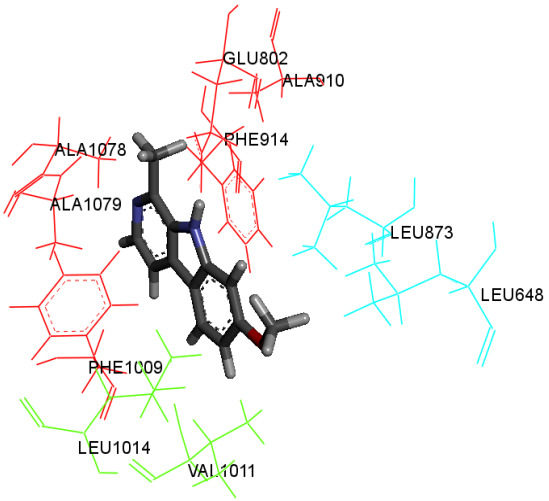


Purified molecules and allopurinol

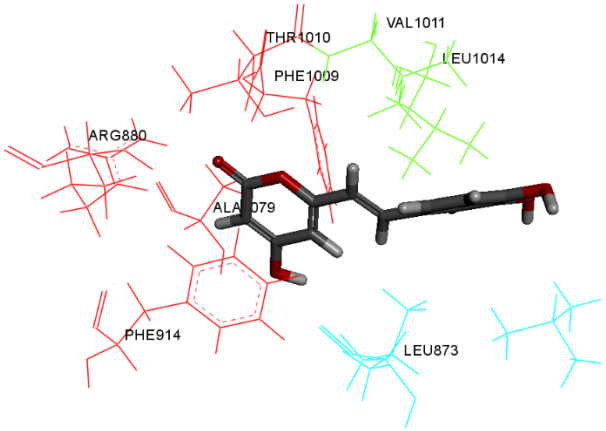
Harmaline



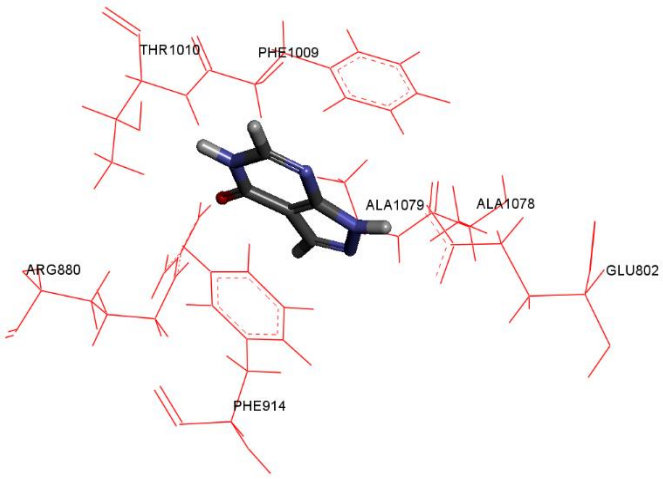
Harmine



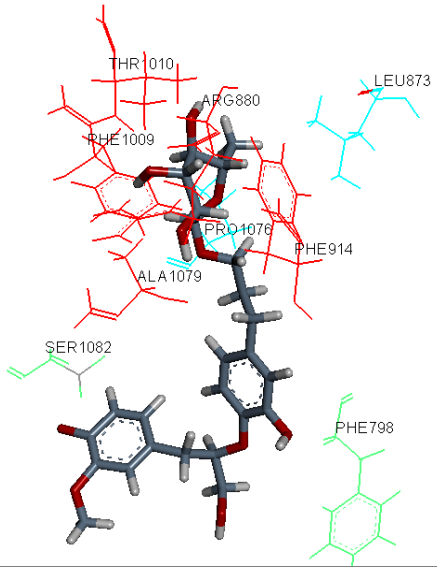
Hispidin



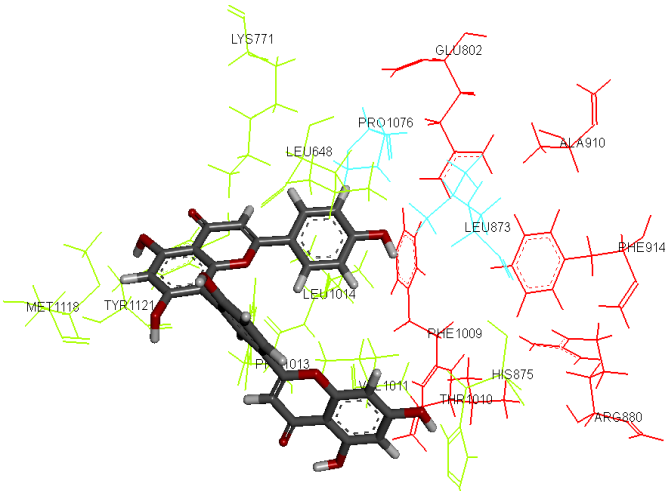
Allopurinol



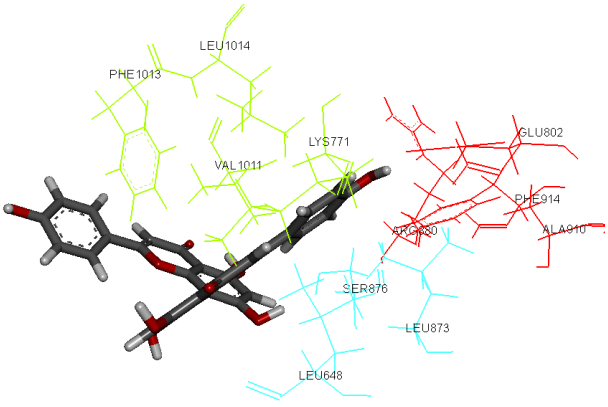
Cp1



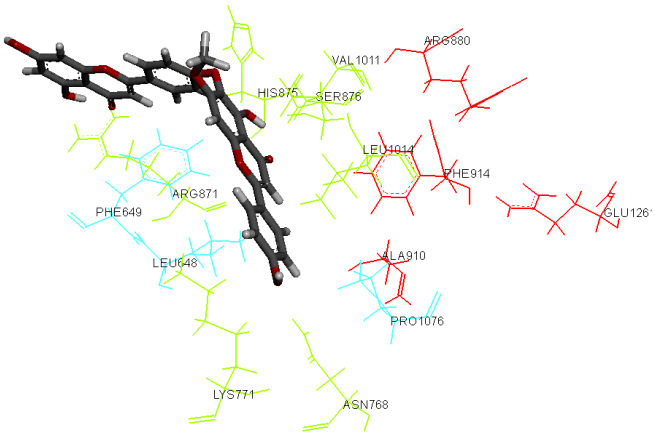
Cp2



Cp3



Cp4



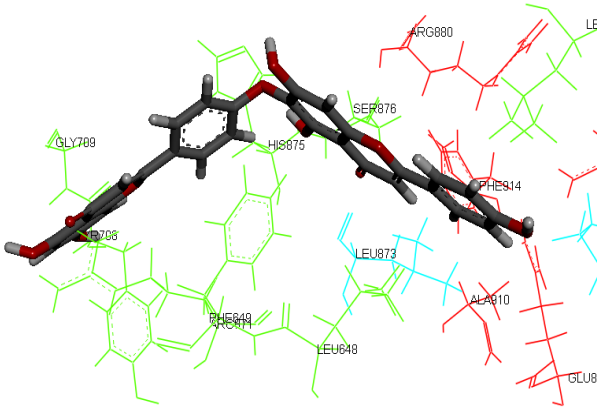
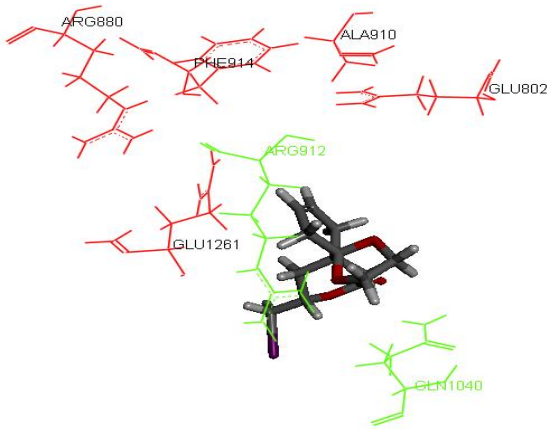
Cp5	Cp6
	

Figure 19. The best pose docking of the studied ligands
(The catalytic site amino acids are presented in lines and colored in red, the binding site amino acids are presented in lines and colored in blue, the contributed amino acids are presented in lines and colored in green).

II. Pharmacokinetic analysis

The drug-likeness rules and the ADMET parameters were calculated and verified for qualification with their standard ranges.

1. Drug-likeness rules

The CMC rule was resumed in the log P (should be between -0.4 and 5.6), molecular weight (MW) (160 to 480 g/mol), molar refractivity (MR) (40 to 130 m^3mol^{-1}), and the total number of atoms (20 to 70 atoms) **[253]**. The LEAD rule was resumed in the molecular weight (must be ≥ 350 g/mol), lipophilicity (Clog P must be < 3), and binding affinity (must be under 0.1µM) **[254]**. The MDDR rule parameters are the number of rings (should be ≥ 3 rings), the number of rigid bonds (should be ≥ 18), and the number of rotatable bonds (should be ≥ 6) **[255]**. The Lipinski rule of five drug-like characters was the hydrogen bond donors (must be ≤ 5), the hydrogen bond acceptor (must be ≤ 10), molecular weight (Should be ≤ 500 g/mol) and ClogP (must be ≤ 5) **[241]**. The molecules that have met the maximum criteria are considered qualified.

The results are summarized in table17.

Table 17. The compounds drug-likeness properties.

	CMC like Rule ¾ considered qualified		**Lead like Rule** $^{2}/_{3}$ considered qualified		**MDDR like Rule** $^{2}/_{3}$ considered qualified		**LIPINSKI Rule** $^{3}/_{4}$ considered qualified	
Inhibitors	**Violation Fields**	**Violations**	**Violation Fields**	**Violations**	**Violation Fields**	**Violations**	**Violation Fields**	**Violations**
Allopurinol	All	4	/	0	N. of Rings, N. Rigid bonds, N. Rotatable bonds	3	/	0
	Not qualified		Qualified		Not qualified		Qualified	
Vitamins								
Vitamin E	/	**0**	Clog P, MW	**2**	**N. of Rings**	**1**	/	0
	Qualified		Not qualified		Qualified		Qualified	
Vitamin B9	/	**0**	MW	1	/	**0**	/	**0**
	Qualified		Qualified		Qualified		Qualified	
Vitamin D3	Log P, N. Total atoms	2	Clog P, MW	2	/	0	Clog P	1
	Not qualified		Not qualified		Qualified		Qualified	
Vitamin C	Log P, MR	2	/	0	N. of Rings N. of Rotatable bonds	1	/	0
	Not qualified		Qualified		Qualified		Qualified	
Purified molecules								
Harmaline	/	0	/	0	N. Rotatable bonds	1	/	0
	Qualified		Qualified		Qualified		Qualified	
Hispidin	/	0	/	0	N. Rings, N. Rotatable bonds	2	/	0
	Qualified		Qualified		Not qualified		Qualified	
Harmine	/	0	/	0	N. Rotatable bonds	1	/	0
	Qualified		Qualified		Qualified		Qualified	
***Cupressus sempervirens* purified molecules**								
Cp1	MW	1	MW	1	/	0	N. H-bond donors,	1
	Not qualified		Qualified		Qualified		Qualified	
Cp2	MW + MR	2	Clog P, MW	2	N. Rotatable bonds	1	MW, N. H-bond donors	2
	Not qualified		Not qualified		Qualified		Not qualified	
Cp3	MW + MR	2	Clog P, MW	2	N. Rotatable bonds	1	MW, N. H-bond donors	2
	Not qualified		Not qualified		Qualified		Not qualified	

Cp4	MW + MR	2	Clog P, MW	**2**	N. Rotatable bonds	**1**	MW	1
	Not qualified		Not qualified		Qualified		Qualified	
Cp5	MW+ MR	2	Clog P, MW	**2**	N. Rotatable bonds	**1**	MW	1
	Not qualified		Not qualified		Qualified		Qualified	
Cp6	/	**0**	MW	**1**	N. Rotatable bonds	**1**	/	0
	Qualified		Qualified		Qualified		Qualified	

N: number **MR**: molar refractivity **MW**: molecular weight **H**: hydrogen

2. ADMET

Compounds have high permeability Caco-2 are highly absorbable; it should be greater than 20. Human intestinal absorption (HIA) of less than 30% is considered poorly absorbed it should be in the range of 80-100%. Water solubility depends on the nature of the structure, temperature and concentration. Compounds that are substrates of P-glycoprotein indicate high levels of absorption. Compounds are considered to have relatively high skin permeability if the log Kp is less than -2.5. Blood-brain barrier penetration (BBB) must be greater than 2. BBB < 1 indicates that the compounds easily cross the blood-brain barrier. The permeability of MDCK cells is classified as follows: low (< 1 nm/s), moderate (1-10 nm/s) and high (>10 nm/s).

High xenobiotics plasma protein binding indicates long half-life in the blood, this serves to extend their effects: 80 to 100 % is considered high, 50 to 80 % (moderate), < 50 % (low).

Cytochrome P450 inhibition can cause unanticipated adverse reactions; our compounds should be a substrate rather than inhibitors. The compound different excretion routes indicate high elimination from the body. The Ames test is considered dangerous if the drug is mutagenic or positive as it is capable of inducing mutations in DNA, the carcinogenicity test must be negative in mice and rats, the compounds used are considered dangerous if they are capable of inhibiting the HERG channel. The ADMET of each compound were predicted and presented in two separate tables (18 and 19).

Table 18. The vitamins and the purified molecules ADMET properties.

Pharmacokinetics	Vitamin E	Vitamin B9	Vitamin D3	Vitamin C	Harmaline	Hispidin	Harmine	Allopurinol
Absorption								
Caco-2 cell permeability (nm/sec) **> 20**	29.11	17.003	54.66	2.48	42.25	10.26	42.21	16.52
Human intestinal absorption (HIA %) **80 to 100 %**	97.83	23.33	100.00	33.15	91.80	85.70	92.82	78.26
Water solubility (mg/l)	-	953.59	-	200764.5	6872.49	1480.75	2520.51	89.04
P-glycoprotein inhibition **a substrate of it indicates high levels absorption**	Inhibitor	Non	Inhibitor	Non	Inhibitor	Non	Non	Non
Distribution								
Blood-brain barrier penetration (C.brain/C.blood) **> 2 cross the blood–brain barrier easily**	19.90	0.049	20.22	0.11	4.53	0.22	3.79	0.18
MDCK cell permeability (nm/sec) **Low (< 1 nm/s), moderate (1-10 nm/s), and high (>10 nm/s)**	38.90	4.83	53.29	0.88	318.01	29.95	317.19	1.57
Plasma protein binding (%) **80 to 100 % is considered high, 50 to 80 % (moderate), < 50 % (low)**	100	48.51	98.25	5.30	62.08	91.36	64.05	3.11
Skin permeability (logKp, cm/hour) **< -2.5 considered high permeable**	-0.515	-4.69	-0.617	-5.14	-4.36	-4.07	-4.38	-5.37
Metabolism								
Cytochrome P450 2C19 inhibition	Inhibitor	Non	Inhibitor	Non	Inhibitor	Inhibitor	Inhibitor	Inhibitor
Cytochrome P450 2C9 inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
Cytochrome P450 2D6 inhibition	Non	Non	Non	Non	Non	Non	Non	Non
Cytochrome P450 2D6 substrate	Non	Non	Non	Non	Non	Non	Non	Non
Cytochrome P450 3A4 inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
Cytochrome P450 3A4 substrate	Substrate	Weakly	Substrate	Weakly	Weakly	Non	Non	Non
Excretion								
Urine	+	+	-	+	+	+	+	+
Feces	+	+	+	-	+	+	+	+
Toxicity								
Ames test	Non-mutagen	Mutagen	Mutagen	Mutagen	Mutagen	Mutagen	Mutagen	Mutagen
Carcinogenicity (Mouse)	Negative	Negative	Positive	Negative	Positive	Positive	Positive	Negative
Carcinogenicity (Rat)	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
HERG_inhibition	Low risk	High risk	Low risk	Low risk	Medium risk	Medium risk	Medium risk	Low risk
TA100 (+S9)	Negative	Positive	Negative	Negative	Positive	Negative	Positive	Positive
TA100 (-S9)	Negative	Negative	Negative	Positive	Positive	Negative	Positive	Positive
Ames TA1535 (+S9)	Negative	Negative	Negative	Positive	Positive	Positive	Positive	Positive
Ames TA1535 (-S9)	Negative	Positive	Negative	Positive	Positive	Negative	Positive	Positive

HERG: Human ether related gene channel, **MDCK:** Mandin Darby Canine Kidney, **Caco-2:** Human colorectal carcinoma, **Kp:** skin permeability constant

Green: safe, Red: dangerous, Orange: medium risk

The *Cupressus sempervirens* ADMET results are presented in table 19.

Table 19. The *Cupressus sempervirens* ADMET properties.

Pharmacokinetics	Cp1	Cp2	Cp3	Cp4	Cp5	Cp6
Absorption						
Caco-2 cell permeability (nm/sec) **> 20**	10.15	12.87	11.68	4.78	4.34	22.06
Human intestinal absorption (HIA %) **80 to 100 %**	59.19	81.19	81.19	91.18	86.95	97.88
Water solubility (mg/l)	233.74	0.14	0.36	0.076	0.197	362.35
P-glycoprotein inhibition **a substrate of it indicates high levels absorption**	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Non
Distribution						
Blood-brain barrier penetration (C.brain/C.blood) **> 2 cross the blood–brain barrier easily**	0.070	0.11	0.133	0.117	0.11	1.305
MDCK cell permeability (nm/sec**)** **Low (< 1 nm/s), moderate (1-10 nm/s), and high (>10 nm/s)**	0.129	0.04	0.043	0.04	0.04	0.50
Plasma protein binding (%) **80 to 100 % considered high, 50 to 80 % (moderate), < 50 % (low)**	75.18	100.00	100.00	100.00	100.00	55.06
Skin permeability (logKp, cm/hour) **< -2.5 considered high permeable**	-3.74	-3.43	-3.39	-2.94	-3.36	-3.48
Metabolism						
Cytochrome P450 2C19 inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
Cytochrome P450 2C9 inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
Cytochrome P450 2D6 inhibition	Non	Non	Non	Non	Non	Non
Cytochrome P450 2D6 substrate	Non	Non	Non	Non	Non	Non
Cytochrome P450 3A4 inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
Cytochrome P450 3A4 substrate	Weakly	Weakly	Weakly	Non	Weakly	Substrate
Excretion						
Urine	+	+	+	+	+	+
Feces	+	+	+	+	+	+
Toxicity						
Ames test	Non mutagen	Mutagen	Non mutagen	Mutagen	Mutagen	Mutagen
Carcinogenicity (Mouse)	Negative	Negative	Negative	Positive	Negative	Positive
Carcinogenicity (Rat)	Negative	Positive	Negative	Positive	Positive	Positive
HERG_inhibition	High risk	Medium risk	Medium risk	Medium risk	Medium risk	Low risk
Ames TA100 (+S9)	Negative	Negative	Negative	Negative	Negative	Positive
Ames TA100 (-S9)	Negative	Negative	Negative	Negative	Negative	Positive
Ames TA1535 (+S9)	Negative	Negative	Negative	Negative	Negative	Positive
Ames TA1535 (-S9)	Negative	Negative	Negative	Negative	Negative	Positive

HERG: Human ether related gene channel, **MDCK:** Mandin Darby Canine Kidney, **Caco-2:** Human colorectal carcinoma, **Kp:** skin permeability constant

Green: safe, Red: dangerous, Orange: medium risk

Discussion

In vitro study

I. Bovine milk and plant material extraction

The obtained bovine milk xanthine oxidase extraction yield was compared with some of the few reported studies, the results were slightly different **[221]**, the majority proceeds with the full purification of it, as a result no studies were found in this context. This variation in yield is normal due to several factors, including the cow's breeds and region, conditions of the extraction, purification, nutrition quality, and climates. The local Algerian bovine breeds, known as "Atlas brown", are small animals that are well adapted to both difficult climatic conditions and limited food resources, especially in Saharan and semi-arid areas as in Laghouat, this can be crucial for the milk quality and furthermore in the enzyme yield and activity **[256]**. The latter is conserved in the bovine milk with 60 % **[257]** due to the presence of molybdenum reserve, our assays revealed that the bovine milk enzyme activity can be conserved for one month in – 30 °C.

The results show that *C. sempervirens* from northern Algeria was mainly rich in flavonoids with a yield of 1.2% following the cited extraction protocol, it shows a high yield compared to Romani *et al* with a yield of 0.7%, in Italy **[258]** and Al-Snafi *et al* with a yield of 0.22%, in Iraq **[259]**, thus, the Algerian flora could be a vital source of flavonoids from *C. sempervirens*.

II. Enzyme activity

1. Effect of some solvent on the enzyme activity

Organic solvents are mandatory in the numerous catalysis, it can causes minor structural perturbation, our results show that DMSO **[35]** and methanol affect the appearance of uric acid in certain percentage level over 25 % and 10 %, respectively. However, DMSO is more effective in dissolving inhibitors than other solvents, ethanol and the phosphate buffer were found suitable. Sodium hydroxide and sulfuric acid were used to dissolve the xanthine substrate (> 0.06 g/l) as recommended by Sigma Aldrich, the results show that both solvents affected the

overall uric acid production even in small percentage (< 5 %), the xanthine must be dissolved in less than 1 % in 1 M of NaOH solution to avoid possible structural perturbation. In our experimental protocol, we decided to use phosphate buffer and DMSO (less than 10 %) to study and determine the kinetic parameters.

2. Determination of the kinetic constants (K_m and V_{max})

The observed substrate activity profile follows the popular Michaelis-Menten form, the resulting graph is a hyperbolic form. According to our results, the xanthine affinity to BXO was presented with a K_m value of 163.55 µM with a V_{max} of 37.59 U. Egwim *et al.*, **[260]** tested the xanthine oxidase from Nigerian Cows (CXO) and Goats (GXO) milks extracts, their results show a K_m value of 8.6 10^{+3} µM and 43.6 10^{+3} µM for BXO and GXO respectively. Moreover, Baghiani team **[261]** have purified cow's milk xanthine oxidase, the milk was obtained from a farm in the region of Setif, Algeria, their results show lower values for both K_m (2.15 µM) and V_{max} (1.83 U) compared to our results. Another studies accomplished by Lewis *et al* **[262]** and Catignani *et al* **[263]** with mammalian and rabbits xanthine oxidase, their results show a K_m value of 6 µM and 22 µM, respectively. The K_m is the concentration of substrate that allows the enzyme to reach half V_{max}; it predicts whether the velocity of product formation will be affected by the supply of the substrate.

Clearly, the source, region, food, and weather conditions of obtained milk affect the kinetic parameters; the identified kinetic parameters confirm that both cow milks from different regions have different characteristics. Less K_m value means high affinity related to more stable ES complex in the active site which indicating a tight binding mode, while a high K_m indicates a less affinity related to less stable ES complex which indicates weak binding in the active site (it takes more substrate to get to V_{max}).

Further, high V_{max} means high K_{cat} (turnover number) defined as the number of substrate molecules transformed per minute by a single enzyme molecule, in our case, the obtained V_{max}

is lower compared to both Nigerian cows and goats milks, this clearly indicates that their ES complex may dissociates fast, our ES complex dissociate slower and that's due to the enzyme concentration in the crude extract, this was confirmed after all the substrate molecules were converted to uric acid in a period of 40 min, the usual reaction is 30 minutes maximum with another cow's milk **[264, 265]**, however this changes did not interfere the results, the only limitation factor in this assay was the time. This is also confirmed with previous studies of the xanthine oxidase in different species **[261]**.

Another limitation linked with low molybdenum (Mo) content **[266]** since it is the key metal in the reaction catalysis (figure 2), the oral administration of sodium molybdate is ignored in some farms due to its cost/availability. According to Hart *et al.*, **[267]**, the molybdenum content affect the activity of some ruminants milk enzymes, same results was found by Baghiani team in the sheep's milk xanthine oxidase.

3. Inhibition activity

XO inhibition assay usually performed with two methods; the spectrophotometric method **[268]** measuring the amount of uric acid released at 292 nm and the HPLC method **[269]**. The first method overestimates the uric acid content since xanthine and uric acid have the same lambda value (290 nm), the second method is efficient and accurate, however, also costly and time consuming. In this thesis, we used the spectrophotometric method with double enzyme detection at 492 nm **[35]** to measure the uric acid concentration.

3.1. Vitamins

According to our results, the used vitamins were potent against the BXO enzyme, compared to the control (table 15), the standard reaction mechanism for xanthine oxidase is based on the nucleophilic attack of the molybdenum hydroxyl system (Mo-OH) on the carbon 8 of the xanthine **[36]**. Vitamin E hydroxyl (O-H) group known by the most reactive function ported on its chroman ring (figure 5) plays an important role in the antioxidative activity by donating a

hydrogen atom to the free radicals and minimize their damage, the chroman ring plays the same role of the allopurinol pyrazole ringside by reacting with catalytic amino acids and Mo atom to ensure the necessary electrons transfer and lead to blocking the active site and that explains its BXO inhibition potency. Studies on vitamin E XO inhibition was found in 1953 by Dinning *et al.*, **[270]**, and Catignani *et al.*, **[271]** proved that the XO activity increases ten times in the liver of rabbits deficient in vitamin E. In 2012, two studies were accomplished by Mohd *et al.*, **[272]** and Ghaffari *et al.*, **[273]**, the first study proves the effectiveness of palm vitamin E XO inhibition in rats exposed to water-immersion restraint stress (WIRS) **[272]**. The second study tested the effects of vitamin E and selenium supplementation on rat XO inhibition **[273]**, they proved that taking oral vitamin E (300 mg/kg) decreased significantly the XO activity **[273]**.

Vitamin B9 is a 2-amino-4-hydroxy-pteridine along with p-aminobenzoic acid main chains can promote hydrogen atoms from multiple sides that could be important to react with Mo atom and block the active site. Takeshi *et al* **[274]** proved our results, and folic acid was effective against the mammalian xanthine oxidase (MXO). We observed that all the cyclic-based inhibitors aromatic or non-aromatic give an important BXO inhibition like vitamin B1, B6, and B7, we confirmed our results with a group of Egyptian researchers **[275]** tested the effect of known plant extract rich in vitamin B group on the rat XO inhibition, the uric acid levels decreased significantly. The rings may act as a nucleophile and donate a hydrogen atom to promote the Mo needs of hydrogen.

Vitamin C is a dihydroxyfuran with four-hydroxyl functions and one ketone; it can promote hydrogen atoms from multiple sides that could be important in any enzyme reaction. In our case, it allows reacting with Mo atom and then inhibiting the BXO with IC50 of 39 µM, compared with the control. Our results were confirmed in many studies (table 8) like Liu *et al.*, **[276]** tested its oral administration in relation to serum uric acid levels, the results of this study show that vitamin C supplementation had a significant effect of decreasing serum uric acid

concentration. Azzeh *et al* **[277]** did the same study with vitamin C oral administration of 500 mg per day, it show a significant decreeing UA levels in hyperuricemic patients.

Vitamin D3 as the most active form in the vitamin D family, it derived from the 7-dehydrocholesterol found in the skin, it holds only one hydroxyl function and four methyl groups in a cyclic-based system, the vitamin D3 could not be a good inhibitor due to its structure nature (high steric hindrance), the only hydroxyl function may be not sufficient to reach the molybdenum center found deep in the active site, the obtained results show weak inhibition with an IC50 of 100 µM, compared with the control; our results were confirmed by Takahashi *et al* **[278]** their study consist of measuring the serum concentrations of 1,25 (OH) 2-vitamin D3 in patients with gout, their results suggest that uric acid high levels may directly decrease serum 1,25 (OH) 2-vitamin D3 in patients with gout by inhibiting 1α-hydroxylase activity.

3.2. Purified molecules

The alkaloids from *Peganum harmala* show high BXO inhibition compared to the control (allopurinol). The chemical structures and molecular weight are crucial in the enzyme inhibition. The tested alkaloids such as harmaline and harmine are reactive groups due to their ring system; they maintain the electrons necessary for the XO inhibition mechanism to block uric acid synthesis.

According to the recorded IC50 values, hispidin was more effective BXO inhibitor than harmine and harmaline. Hispidin has two benzene rings; as a result, they promote electron needs for the reaction mechanism. In the literature, we did not find BXO inhibition studies for the tested purified molecules; we are the first in this context.

3.3. *Cupressus sempervirens* extracts

The extracts from *C. sempervirens* show high BXO inhibition according to the IC50 values and compared to the control (allopurinol). Since the acetate ethyl and chloroform fractions were both sources of flavonoids and alkaloids; as a result, the inhibition activity was effective due to

this metabolites O-H functions as the hydrogens donors which are crucial for the BXO inhibition mechanism **[36]**, we suggest that hydroxyl function reacts with nucleophilic amino acids like GLU1261 to donate a hydrogen atom to reduce the molybdenum hydroxyl system and form a covalent bond ends with blocking the active site.

4. Inhibitory kinetics

In case of competitive inhibition using Dixon method **[222]**, the data for each substrate concentration fall on straight lines that may intersect above the X-axis (on the negative side of the graph) or at I = -Ki and $1/v = 1/V_{max}$, in competitive inhibition, K_m apparent increases relative to K_m while V_{max} does not change, hispidin (Appendix: 06) was found competitive BXO inhibitor (table 15), this type of inhibition occurs when the inhibitor binds to the enzyme at the substrate site, resulting in blocking the complex [ES] formation, in this case the velocity depends often on the K_m.

Similarly, the straight lines may also intersect above the x-line on the negative side of the graph, which is the case for a mixed-type non-competitive inhibitors **[279]**, similar to competitive inhibition, however, it depends on V_{max} to differentiate them **[280]**, in the case of non-competitive inhibition; the inhibitor binds to the enzyme at a site other than the active site and independently of the substrate binding. Nevertheless, this inhibitor binding makes the [ES] complex inactive, the K_m remains unchanged and V_{max} changes (it decreases), that is the case of the control (allopurinol), harmaline, harmine, and all tested vitamins (Table 15, Appendix: 06).

In drug discovery, the choice of a competitive or non-competitive inhibitor depends on whether the intracellular concentration of the required substrate or product is increasing or decreasing **[281]**, and then a competitive or non-competitive inhibitor will function, since both will inhibit the utilization of the substrate, resulting in its accumulation. If the requirement is to decrease the intracellular concentration of the product, then the inhibitor must be non-

competitive. If the requirement is to decrease the intracellular concentration of the substrate then the inhibitor must be a competitive inhibitor, and the result will be stable rate of product formation **[281]**.

4.1. Vitamins

Vitamins were found non-competitive inhibitors, fat soluble vitamins (D3 and E) reduce the BXO velocity relatively the same as water soluble vitamins (B9 and C), however, water soluble vitamins were effective with small doses (IC50 < 40µM) which may increase their intake doses during the day with no risk since their storage in the human body is weak **[86]**, fat soluble vitamins would serve as long treatment since their storage is relatively higher **[69]**, it may be used in some chronic cases or in the first hyperuricemia pics. Studies on the bovine xanthine oxidase kinetics with our tested inhibitors were not found, our study is the first in this context. However, other xanthine oxidase sources were tested.

Vitamin B9 was assayed by Lewis *et al* **[282]**, he proved that vitamin B9 and its metabolites, tetrahydrofolic acid, 5-formyl-tetrahydrofolic acid, and 2-amino-4-hydroxypteridine were effective against bovine spleen xanthine oxidase with a Ki of 0.5 µM, 1.25 µM, 13 µM, and 1.25 µM, respectively and 4.88 µM for allopurinol. According to our study, vitamin B9 was a non-competitive inhibitor with a Ki of 15 µM compared to allopurinol with 6.5 µM, this difference is due to the used XO source.

Vitamin E studies toward xanthine oxidase were found however, kinetic studies were not found, the majority were testing its inhibition effect *in vivo* using rats **[273]**, rabbits **[271]**, and cattles **[283]**. The studies agree that deficiency in vitamin E leads to increase the serum levels of the XO, our results confirm this finding, the obtained Ki was 29 µM with non-competitive inhibition type (table 15).

Vitamin D3 serum concentration is low in gout patients, allopurinol oral administration releases vitamin D3 storage in the body **[284]**. Our results show moderate inhibition with Ki

value of 20 µM (table 15). Suyun *et al* **[285]** tested its inhibition effect with vitamin B2 on the pure BXO, their results shows competitive inhibition with K_m value of 27 µM, suggesting using it as an alternative treatment to reduce hypovitaminosis D3 and treat gout especially in older patients.

Vitamin C was not well studied concerning its inhibitory kinetics. Researchers studied its positive effect towards the serum uric acid levels **[191]**, it can reduce it significantly, and our study confirms this finding with a Ki value of 12 µM (table 15), additionally, vitamin C ranked first among the vitamins with non-competitive inhibition type.

4.2. Purified molecules

Harmine is the best inhibitor among all tested compounds in this thesis, it acts also as blocker in another site rather than the active site (non-competitive inhibitor), its Ki value was better than the control (2.5 µM), studies on its BXO inhibition was not found. This is the originality of our work. Harmine is a well known compound as potent inhibitor to different receptors and enzymes **[286, 287]**.

Harmaline was found effective non-competitive inhibitor, it reacts like harmine; in another site rather than the active site, velocity was slowed and the Ki value was 11 µM, in the literature, we did not find its BXO inhibition.

Hispidin is a competitive inhibitor, it blocks the BXO active site with Ki value of 3.5 µM, studies on its BXO inhibition were not found, we tested its inhibition through our previous works **[97, 288-291]**, in *Candida rugosa* lipase, it shows a Ki value of 0.27 mg/ml with competitive inhibition type **[291]**, it is also a well-known compound as potent inhibitor to different enzymes **[292]**.

In silico study

According to the *in vitro* study, the tested compounds show a significant inhibition towards BXO. In order to obtain a deep knowledge on its action mechanism, we performed a molecular docking studying the pure compounds as ligands from each source with determining the interactions type and the involved amino acids.

The BXO enzyme is a small protein (~ 273.91 kDa) with two chains named A and C (shown in figure 1), the C chain contains the active site, we identified the amino acids interacted with each inhibitor, six of the eight amino acids form the pocket, which explains the high affinity towards BXO, 70% of the saved interactions were hydrophobic Alkyl, Pi-Alkyl, Pi-Sulfur, Pi-Pi Stacked and Pi-Pi T-shaped.

These interactions reveal the hydrophobic environment within the active site, and probably inhibitors with aromatic rings with molecular weight less than 500 g/mol **[241]**, can serve as good BXO inhibitors.

I. Molecular docking

1. Vitamins

Vitamin E and folic acid were recorded as the best inhibitors with the highest PLPchem score among the other inhibitors (table 16). These results were proved by the *in vitro* study. The used PLPchem score is an empirical fitness function optimized for pose prediction used to model the steric complementarity between protein and ligand **[234]**. We focused on the hydrophilicity and hydrophobicity characters to define the inhibition mechanism of the ligands towards BXO. Thus, the active site of BXO is mainly formed by hydrophobic amino acids and minor hydrophilic amino acids, which explains the formation of donor/acceptor hydrogen bonds.

1.1. Vitamin E

Vitamin E was used as inhibitor model to demonstrate the inhibition mechanism, in BXO; the active site is divided into two parts, the catalytic site, and the binding site (appendix 08).

The reaction initiates first in the binding site observed on the external side and formed with non-polar amino acids like; Leu648, Phe649, Leu873 and Pro1076. These amino acids form hydrophobic interactions (alkyl type) with its chroman ring, vitamin E hydrophobic tail is the leadership, enters first and deep in the catalytic site formed by Arg880, Glu802, Thr1010, Phe914, and Glu1261 and block the BXO active site, the complex is stabilized by the chroman ring hydroxyl group which donate a hydrogen atom and form strong bridge salt with Leu873.

The preferred orientation of the vitamin E to enter inside the active site was similar to his native action that allows for penetration into biological membranes **[293]**.

1.2. Vitamin B9

Folic acid saved 18 favorable interactions and recorded the best hydrogen bond number among all tested vitamins with a distance of 1.73Å with Met1038. The folic acid structure is composed of three parts; 2-amino-4-hydroxy-pteridine (pterin), p-aminobenzoyl group, and glutamic acid.

The pterin ring enters first inside the active site forming a strong hydrogen interaction with Met1038, p-aminobenzoyl group was the most reactive group according to the electron density map, it interacts with the binding site amino acids such as Leu648, Phe649, Leu873, Pro1076, Phe914, Phe1009, and Ala1079. Val1011 α-amino group reacts with p-aminobenzoyl ketone function in the binding site forming a hydrogen interaction and blocks the active site.

1.3. Vitamin D3

Cholecalciferol or vitamin D3 was recorded as the weakest inhibitor to BXO, with the lowest saved PLPchem score (table 16), which was explained by the nonpolar nature of vitamin D3, all poses saved were in the binding site far from any catalytic amino acids, only the hydroxyl function was pointed to the catalytic site as it is the only possible way to enter the catalytic site with unfavorable interaction choice. All other saved interactions were hydrophobic (alkyl type).

1.4. Vitamin C

Ascorbic acid saved seven favorable interactions, most of them were hydrophilic type by its (R)-3,4-dihydroxy-5-methylfuran-2(5H)-one side chain, we suggest that its hydroxyl functions ported in the ethane-1,2-diol side was responsible on its orientation inside the active site by forming four hydrogen bonds, the minimum saved distance was 1.97Å with Arg880. The ethane-1,2-diol enters first inside the active site then stabilizes in the catalytic site by Arg880, ascorbic acid was found deeply in the active site (exactly in the hydrophilic part side of the active site), surrounded with catalytic site amino acids like; Thr1010 and Arg880, binding amino acids like; Phe914, and Ala1079. The vitamin C molecular weight and presence of hydroxyl functions were crucial on its mechanism of action.

2. Purified molecules

2.1. Harmaline

Harmaline is a heterocyclic compound isolated from *Peganum harmala*, its structure centered by the indole ring attached with tetrahydropyridine and benzene ring. From structural view, harmaline was docked in straightly and deeply in the active site, its RR value was 100%, this may explain its affinity to BXO. However, its PLPchem score was lower than other inhibitors (table 16). To understand its mode of action, we localize the most reactive function in harmaline structure, which is the indole ring, it was pointed to the catalytic site and formed hydrogen bond with Glu802 hydroxyl function, it forms seven more interactions with hydrophobic amino acids like Phe914, Ala1079, and Leu648. In total of 21 favorable interactions, 17 were formed with the indole ring, it was responsible for the preferred best orientation inside the active site, as a results, harmaline centralized alongside the hydrophobic amino acids as the preferred type due to it hydrophobicity. Additionally, Phe914 and Phe1009 were the most interacting amino acids with six hydrophobic interactions, type Alkyl, and Pi Alkyl. We suggest that the indole ring was essential in the inhibition mechanism of BXO. The

ligands with indole rings could be very potent inhibitors to BXO. Ala 1078 and Ala1079 were interacting with the pyridine side chain and may play a role in stabilizing the harmaline. Leu648 and Leu1014 could be also important in the inhibition mechanism by interacting with the methoxy group ported in the benzene ring.

2.2. Hispidin

Hispidin is a phenolic antioxidant and fungal metabolite isolated from *Inonotus hispidus*, it belongs to the bicyclic compounds (table 12), hispidin maintained a high PLPchem score and ranked within the top models of BXO inhibitors (table 16), to explain its inhibition mechanism, we concentrate on the structure rotatable parts, the side-chain priority of login in the active site, and hydrogen bond number. The ketone function ported in the pyran ring interact with Arg880 and Thr1010 and form three conventional hydrogen bonds, the pyran side chain was found to be the first to enters deep in the active site surrounded by catalytic site amino acids, we identified the rotatable part responsible for the best orientation and was the C6-C7 bond (figure 20) with torsion of 50.41°. The pyran ring was also interacting with Phe914, Leu1014, and Ala1079 forming three hydrophobic interactions type Pi-Pi Stacked and T-shaped. In total, six interactions were formed with the pyran ring. Additionally, the benzene ring was found stacked in the upper portion of the active site with the binding amino acids; Leu648, Leu873, and Leu1014 forming Alkyl and Pi-Alkyl interactions, the two benzene hydroxyl functions found to be not reactive due to their displacement outside the active site.

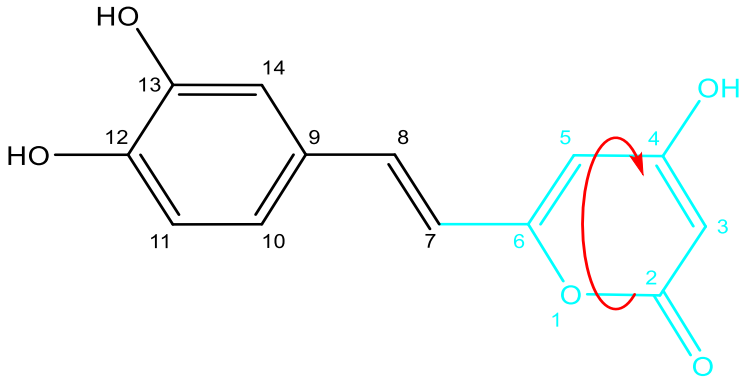


Figure 20. The rotated part (colored in blue) of the best hispidin orientation.

2.3. Harmine

Harmine structure is similar to harmaline with dehydrogenation in the C11=C14 bond, the tetrahydropyridine and anisole rings were linked by an indole, from a structural view harmine take a vertical shape inside the active site, contrary to harmaline. Its RR value was 100% similar to harmaline, however, its PLPchem score was better than harmaline and ranked second after hispidin (table 16). To explain its inhibition mechanism we tracked the same steps mentioned in harmaline. We found that the most reactive function in harmine was also the indole ring in which was pointed deep in the catalytic site and form hydrogen bond with Glu802 hydroxyl function, however, Glu802 was slightly close compared to harmaline (table 16), the harmine structure was centered in the active site surrounded with three amino acids; Phe, Leu, and Ala, the indole ring forms six hydrophobic interactions with Phe914 and Phe1009, the anisole methoxy function interact with Leu648, Leu873, and Leu1014 forming three hydrophobic interactions.

We suggest that Ala910 and Ala1079 can stabilize the pyridine sidechain, as they formed a rigid carbon-hydrogen bond with its amine function, eventually, harmine form 22 favorable interaction, 16 were formed with the indole ring, primarily, the dehydrogenation effect on C=C bond influence significantly on the structure overall movement which lead to relatively lower bond rotation effect; as a result, the C11=C14 bond alter hydrophilic amino acids.

3. Cypress molecules

3.1. Compound 1 (Cp1)

Cp1 is a simple phenol, its best pose was found deep in the active site, the pyranose ring was oriented to the upper part and reacted with the catalytic site amino acids; Arg880, Phe1009, and Phe914 by forming hydrogen (Arg880) and hydrophobic (Phe1009 and Phe914) interactions. This could play an important role in stabilizing the pyranose ring. Both benzenes established four hydrogen bonds, two through methoxy with Gln1194 and Gln1040, and two with Phe798

and Ser1082. Hydrophobic interactions were also found with Arg912, Val1259, Val1081, Ala1078, and Phe914. In summary, the pyranose ring was the most interactive and formed both hydrophobic and hydrogen bond. Therefore, the most saved interactions of Cp1 were favorable type (table 16), demonstrating the high affinity towards BXO.

3.2. Compound 2 (Cp2)

Cp2 is a biflavonoid; its structure includes two flavonoids attached together. MD shows that the best orientation of cp2 was next to the aromatic amino acids of BXO. Cp2 was hooked and locked near the active site, which was evidenced by its RR value and PLPchem score (table 16). Tyr1121 and Phe1013 form hydrophobic interactions with its aromatic rings. The high molecular weight and lipid solubility of Cp2 (table 12) strongly influence its orientation in the active site due to the presence of hydrophobic amino acids in the binding site such as Pro1076, Leu648, Val1011, and Leu873. The structure was stabilized by a covalent sulfur-oxygen interaction between the sulfur function of MET1118 and the hydroxyl carried in the aromatic ring; thus, no conventional hydrogen bond were formed except for the carbon-hydrogen type. We suggest that ligands with a multi-aromatic ring may not be the preferred ligand for BXO due to their affinity for aromatic amino acids outside the active site.

3.3. Compound 3 (Cp3)

Cp3 is an isomer of cp2, its best orientation takes a triangle shape (figure 19), close to the active site and interacting with cyclic amino acids; Phe914. Phe1013 forms three T-shaped Pi-Pi hydrophobic interactions with its benzene and chroman rings. Val1011 and Pro1012 form four Pi-Alkyl type interactions with its second chroman ring. Phe1013 and Val1011 were the most reactive; as a result, 6 interactions were formed, the latter being crucial for the stability of Cp3; consequently, the PLPchem score was slightly better than cp2, with 100% favorable interactions. Leu873 and Leu648 also interacted as described in cp2, proving their importance in the reaction mechanism, Lys771 was the closest (2.16 Å); it could be the nucleophilic amino

acid suggesting that it reacts by donating a hydrogen atom to the ketone function of the chroman ring.

3.4. Compound 4 (Cp4)

Cp4 is the third biflavonoid tested. Cp4 takes a triangular shape like cp3 as the preferred orientation (figure 19), it interacts with the cyclic amino acids; Phe914, Pro1076, and His875, however; it enters the catalytic site with only one flavonoid as the most reactive one compared to the second flavonoid which was oriented outside the active site. Phe649 forms three hydrophobic Pi-Pi T-shaped interactions. Val1011, Leu648, and Pro1012 form six Pi-Alkyl interactions. Leu648 and Phe649 were the most interacting amino acids with five hydrophobic interactions. Its PLPchem score was better than cp2 and cp3; however, its RR value was the lowest due to its high molecular weight, it was bound mainly by hydrophobic amino acids. Cp4 molecular weight (table 12) was the largest among all ligands tested, thus it binds to the entrance and blocks the active site.

3.5. Compound 5 (Cp5)

Cp5 is the fourth biflavonoid tested in this thesis, the structure of Cp5 was similar to cp4 excluding the hydroxylation at C13, the rings are attached by a benzene ring with a C-O-C bond like cp4. Cp5 also takes a triangle shape with a slight slope towards the chroman rings, this slight slope negatively affects the binding mode of Cp5 in the active site; as a result, unfavorable bump-like interactions were found; finally, its RR value was lower than the other biflavoniods. Leu648 and Phe649 were the most interacting with a total of four hydrophobic Pi-alkyl and Pi-Pi T-shaped interactions.

The triangular form preferred by the tested biflavoniods as best pose like cp3, cp4 and cp5 were proved the most stable, however: the high molecular weight can be an obstacle to the complex complementarity inside the BXO active site, the biflavoniods can be a good active site blocker to BXO.

3.6. Compound 6 (Cp6)

Cp6 is a monoterpenoid, it was found to be the weakest inhibitor model of BXO with an RR value of 0% and the lowest PLPchem score compared to other inhibitors (table 16). Cp6 was placed outside the active site, the closest interactions were next to Glu1261, in total two interactions were found, this could be due to its structure poor in functional groups, the only reactive function was the ketone, this also explains its lower PLPchem score, the structure was small, probably it cannot fit the volume of the active site, this proves that the molecular weight plays an important factor in the complementarity in any receptor-ligand complex.

Human and bovine xanthine oxidase molecular docking comparison

After comparing the docking results between BXO and HXO (table 16), we notice similar results regarding the action of both substrates and all inhibitors towards their receptors (BXO and HXO). All docking results saved in BXO were repeated in HXO however, in a decreasing manner and with the same order regarding the percentage of RR, PLPchem score, bond length, and number of favorable/unfavorable interactions. The values recorded in BXO were always high compared to HXO; the hypoxanthine substrate PLPchem score recorded 53.86 (with 11 favorable interactions) and 26.08 (with 8 favorable interactions) for BXO and HXO respectively. The control (allopurinol) PLPchem score recorded 48.88 (with 11 favorable interactions) and 24.88 (with 7 favorable interactions) for BXO and HXO respectively. The same results were relatively found in all inhibitors, although the values saved in BXO were higher than HXO. These results are expected since the active site of BXO is larger than HXO and contains different types of amino acids, including polar and non-polar, making it more accessible than HXO. Ultimately, all compounds tested were found to be potent compared to allopurinol in both BXO and HXO.

II. Pharmacokinetics analysis

1. Vitamins

1.1. Vitamin E

The vitamin E has passed 3/4 druglikeness rules (table 17); the violation was the high molecular weight and lipophilicity. According to the ADMET study, vitamin E was good absorbed in both intestine and caco-2 cell permeability in this case its intestinal permeability was approved (table 18) due to its hydrophobic nature as it is soluble in fat foods, however it shows risk towards the glycoprotein inhibition activity, in this case it cannot penetrate the intestines in some patients. The predicted distribution properties were found acceptable. Additionally, its half-life is long **[294]**; in case of treatment using vitamin E, no necessary high doses are needed. Its predicted metabolism properties were found moderate compared to other compounds, it is found equally substrate or inhibitor for cytochrome enzymes, in this case the effectiveness depends often on the patients status, the elimination routes were varied and the toxicity result were the best of all predicted compounds in this thesis.

1.2. Vitamin B9

The vitamin B9 has passed 4/4 druglikeness rules (table 17), the only violation was its high molecular weight. According to the ADMET study folic acid was not good absorbed in both intestine and caco-2 cell permeability, which were considered as the references *in vitro* to evaluate intestinal permeability (table 18), however it shows advantageous in two other absorption parameters as it is well water soluble and presents no risk towards the glycoprotein inhibition activity, in this case it can penetrate the intestines. The predicted distribution properties were found weak, since its half-life is short **[295]**, it should be taken multiple times a day. The predicted metabolism properties were found strong compared to other compounds, it is found substrate for most cytochrome enzymes, which ensure its rapid effectiveness,

elimination routes were varied and the toxicity result shows mutagenicity in the Ames test and a high risk to the heart.

1.3. Vitamin D3

The vitamin D3 has passed 2/4 druglikeness rules, the violation was the high lipophilicity and the high number of atoms which influence its molecular weight (table 17). According to the ADMET study vitamin D3 was better than vitamin E, it was good absorbed in both intestine and caco-2 cell permeability, in this case its intestinal permeability was approved (table 18), due to its hydrophobic nature it is also soluble in fat foods, however it shows risk towards the glycoprotein inhibition activity, like the vitamin E it cannot penetrate the intestines in some patients. The predicted distribution properties were considered acceptable; from the obtained results. Its predicted metabolism properties were found moderate same as vitamin E, the elimination routes were restricted to only feces route, and the toxicity result were mutagen to Ames test and positive to carcinogenicity, accordingly it can cause mutations in the DNA in long term **[296]**.

1.4. Vitamin C

The vitamin C has passed 3/4 druglikeness rules, the violation was its lower molecular refractivity (table 17). According to the ADMET study, ascorbic acid was weakly absorbed in intestinal tract (table 18), however, it shows advantageous in two absorption parameters as it is highly water-soluble and presents no risk towards the glycoprotein inhibition activity. The predicted distribution properties were found weak, as known that water-soluble vitamins are weakly stored **[295]**, multiple doses a day are mandatory. Its predicted metabolic properties were found to be strong, it is a substrate for most cytochrome enzymes, the majority of properties were found to be similar to those of vitamin B9, the urine route was the only route of excretion, and its toxicity result shows mutagenicity in the Ames test with no risk to the heart, hence it should be taken strictly.

2. Purified molecules pharmacokinetics

Harmaline and harmine passed 4/4 druglikeness rules except for hispidin (in MDDR) and were qualified suitable compounds for drug development (table 17). The toxicity profile of harmaline and harmine was expected and explains the currently observed effects as it belongs to the alkaloid family.

The use of alkaloids is very strict for medical or scientific research purposes. Therefore, alkaloid drugs should be prescribed with caution. Until now, no evidence reports in this context were found, so we could not discuss our work.

2.1. Harmaline

Harmaline predicted ADMET parameters (table 18), show high caco-2 cell permeability, it can permeate easily cell membranes, the blood-brain barrier penetration and intestinal absorption were suitable, it shows also high buffer solubility. However, it can be an inhibitor of glycoprotein activity, its elimination route was variant, and the toxicity profile shows mutagenicity to Ames tests, with medium risk for the human ether related gene channel inhibition. In this case, it can be dangerous for chronic disease patients; it must be taken carefully.

2.2. Hispidin

The predicted ADMET parameters of hispidin (table 18) show lower caco-2 cell permeability and blood-brain barrier penetration, than other inhibitors; however, its intestinal absorption was suitable. Its buffer solubility was lower at least twice compared to harmine and harmaline. However, no glycoprotein inhibition activity was found.

Its elimination route was the same as all inhibitors and shares a similar metabolism profile with other inhibitors; the toxicity profile shows mutagenicity to Ames tests, with medium risk for the human ether related gene channel inhibition. It was relatively negative in other Ames tests and ranked first in this parameter.

2.3. Harmine

The predicted ADMET parameters of harmine (table 18) show suitable caco-2 cell permeability, blood-brain barrier penetration, and intestinal absorption, no glycoprotein inhibitory activity was found, its elimination route is the same as that of all inhibitors, and its metabolic profile is identical to other inhibitors, the toxicity profile shows mutagenicity and positivity in all Ames tests, with a medium risk for inhibition of the human ether gene channel.

3. Cypress compounds

3.1. Cp1

Cp1 has passed 3/4 druglikeness rules (table 17), it was qualified in MDDR, LIPINSKI, and LEAD filters, its total polar surface area value (TPSA) was slightly bigger due to its molecular weight (table 12), Thus, molecules with TPSA greater than 140 Å **[226]** tend to be poor in permeating cell membranes. The predicted ADMET parameters (table 19) show minimal blood-brain barrier penetration and intestinal absorption; however, urine and feces can excrete it; the toxicity profile shows no risk on Ames tests, however, it may present a high risk for human ether-related gene channel inhibition. In this case, it can be dangerous for cardiovascular patients.

3.2. Cp2

Predicted ADMET parameters of Cp2 (table 19) show poor blood-brain barrier penetration. However, its intestinal absorption was suitable. Urine or feces can excrete it. Its toxicity profile was mutagenic in Ames tests, with a medium risk for HERG. The drug-likeness rules showed that Cp2 was only qualified in the MDDR filter (table 17); its TPSA was out of range (>140 Å). Finally, Cp2 passed 1 of 4 drug rules.

3.3. Cp3

Cp3 presented low blood-brain barrier penetration however its intestinal absorption was high; the isometry to cp2 did not change its physicochemical properties, ether urines or feces can

excrete it, the toxicity profile was the best among all tested compounds, no danger or risks on Ames tests or other related tests, with medium risk for HERG; however, its drug-likeness properties showed qualification only in the MDDR filter, its TPSA was the same as cp2.

3.4. Cp4

Cp4 shows also low blood-brain barrier penetration, contrary its intestinal absorption was high compared with other inhibitors, the elimination routes were variant, the toxicity profile shows mutagenicity to Ames test, and Carcinogenicity to both mouse and rats, no danger or risks on the other Ames tests, with medium risk for HERG, its drug-likeness properties showed qualification in the LIPINSKI and MDDR filter, its TPSA was acceptable.

3.5. Cp5

Cp5 shares the same properties as other cypress compounds concerning the low blood-brain barrier penetration, thus its intestinal absorption properties were high (table 19), the metabolism results summarized in cytochrome P450 activity were the same as cp2, cp3, and cp4. Clearly, the biflavoniods class has the same influence on the cytochrome P450, the elimination routes were variant, the toxicity profile shows mutagenicity to Ames test, and carcinogenicity to rats test, no risks for the other Ames tests, with medium risk for HERG, the cp5 have passed 2/4 drug-likeness rules, its TPSA was acceptable.

3.6. Cp6

Cp6 ADMET profiles show low blood-brain barrier penetration as all cypress compounds thus its intestinal absorption was the highest compared to others, similarly, the cytochrome P450 activity was the same as other compounds, the toxicity profile shows positivity to all ames test, and carcinogenicity to both mouse and rats, with low risk for HERG, in this case it can be safe only for the heart, the cp6 drug likeness have passed 4/4 rules (table 17) as the best recorded drug likeness properties.

Until now, no evidence reports in this context were found so, we could not discuss our work.

Conclusion

New inhibitors for bovine xanthine oxidase were discovered through this work depending on the *in vitro* and *in silico* modeling. Natural purified molecules, vitamins, and some extracts from known traditional plants were proved effective against the bovine xanthine oxidase. We evaluated their physicochemical properties through this work by performing molecular docking and SAR studies to define their mechanism of action. Furthermore, the drug like-rules and ADMET parameters as the pharmacokinetics properties were checked to ensure their safety.

Harmaline and harmine showed similar effect towards BXO as two alkaloids belong to the same plant family, hispidin was also effective inhibitor with competitive inhibition type different than harmaline and harmine, their ADMET properties explore the weak and the strong point to be taken into consideration with promising *in vivo* tests, the drug-likeness properties show reasonable qualifications in all most rules, the tested inhibitors may be a powerful treatment for gout, it can be found in many traditional remedies and used in tisane as multi-beneficial effects, the used inhibitors may replace the usual medications to reduce the secondary effects.

The vitamins were ranked second in this thesis as a new treatment strategy, vitamin B9 was the best selected inhibitor of bovine xanthine oxidase it shows the minimum IC50 value among all tested compounds, vitamin E show high levels of safety taking it; is completely safe no risk for any organs it can be taken with small doses with promising results.

The consumption of the recommended daily requirement from vitamin E gain multiple beneficial effect, as anti-inflammatory in the first hyperuricemia pics and as a good natural drug to relieve stress on the kidneys by lowering the serum uric acid levels and increasing the amount of hypoxanthine and xanthine in the bloodstream as better excreted compounds by the kidneys.

Using vitamins protect cells from oxidative damage, prevent other disease like cardiovascular disease and type 2 diabetes, and even protect other vitamins from oxidation by oxygen, this

treatment strategy can reduce gout future symptoms, and especially these tested vitamins exist in small amounts in the gout patient's daily food routine.

We suggest taking water-soluble vitamins in higher doses to be effective due to their weak storage in the body, contrary; the fat-soluble vitamins should be taken with small doses as they last more in the body, which present an advantageous point.

Through this work, we demonstrated a new activity of the novel *Cupressus sempervirens* L. plant extracts as bovine xanthine oxidase inhibitor; the used extract was evaluated from different physio-chemical points supported by strong ADMET properties with highly recommended *in vivo* assays, their drug-likeness properties showed qualification in multiple rules, the *Cupressus sempervirens* have shown a promising approach to treat the hyperuricemia pics, It can be a good natural treatment strategy to reduce inflammation and relieve pain, with no danger on the kidneys and other organs, according to our study, using the purified *Cupressus sempervirens* compounds could replace the use of the known hyperuricemia drugs like the synthesized anti-inflammatories to minimize their side effects.

Uric acid role in oxidative stress is not entirely studied however, a few studies have investigated the role of UA on vascular relaxation; "regarding the antioxidant role of uric acid and suggests that it has an important role as an oxidative stress marker and a potential therapeutic role as an antioxidant" *in vivo* UA is an antioxidant and participate in the overall antioxidant potential. It reduces the damage of peroxynitrite; neutralizes singlet oxygen, and free radicals.

Our perspectives are to confirm the ADMET properties of the pure compounds *in vivo* by performing the first clinical trials with these promising results; however, a strict diet must be applied before to avoid excessive purine production.

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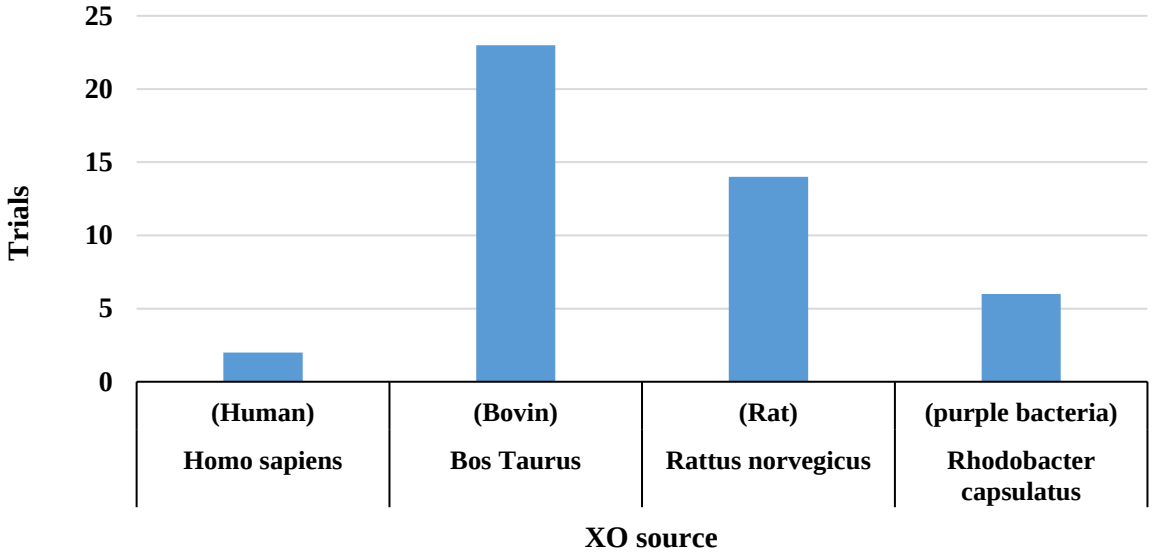
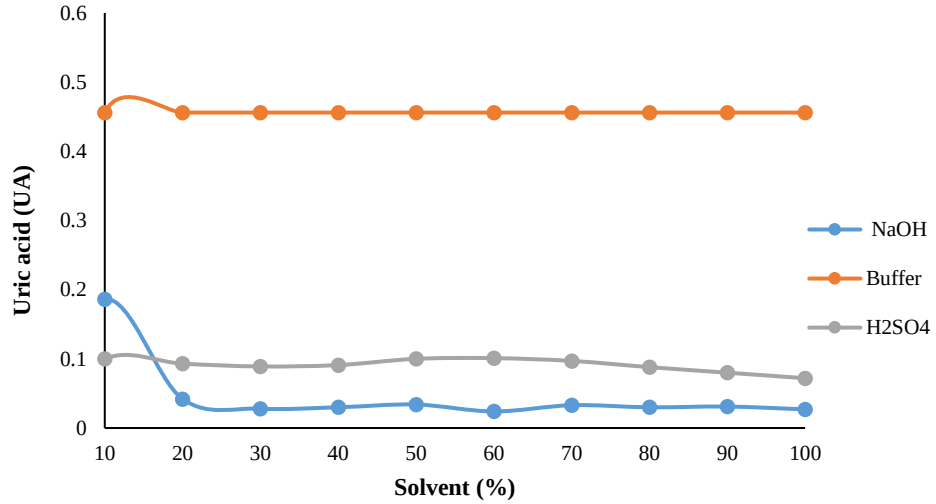
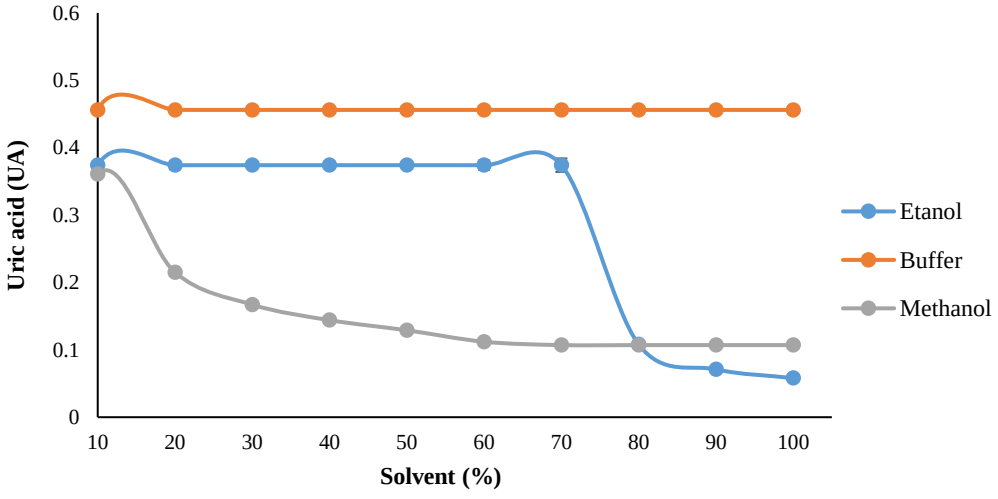
Appendices

Appendix 01: XO available 3D crystal structures (from PDB).



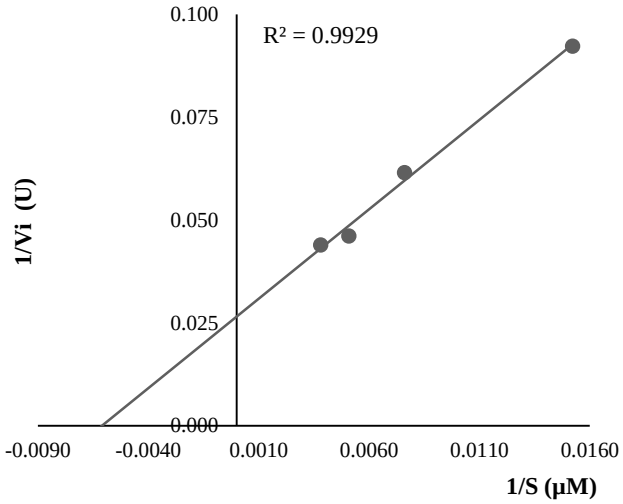
Appendix 02: Uric acid formation in the presence of different solvent percentage.



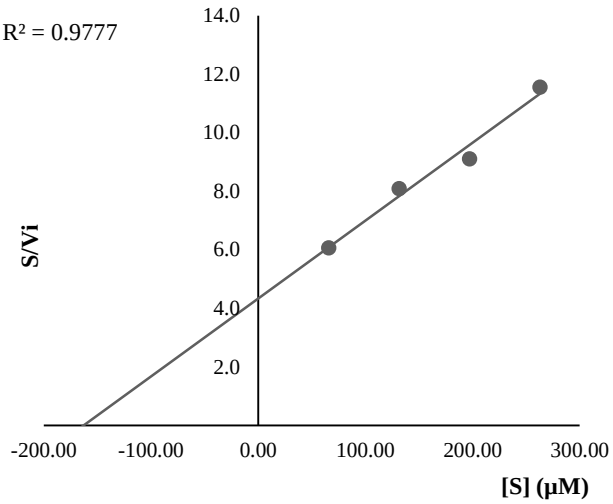


Appendix 03: Line-weaver Burk (A) and Hanes Woolf (B) plots.

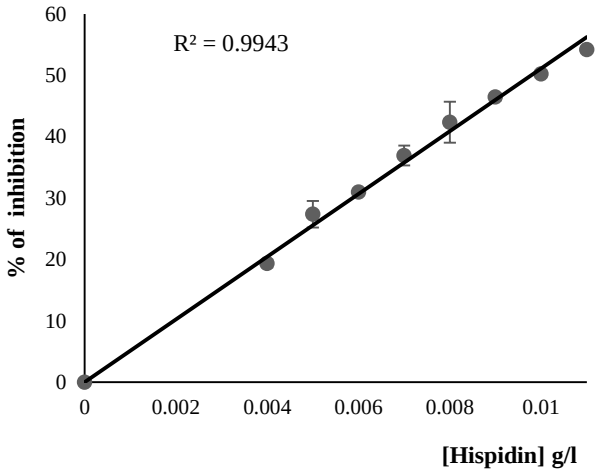
A

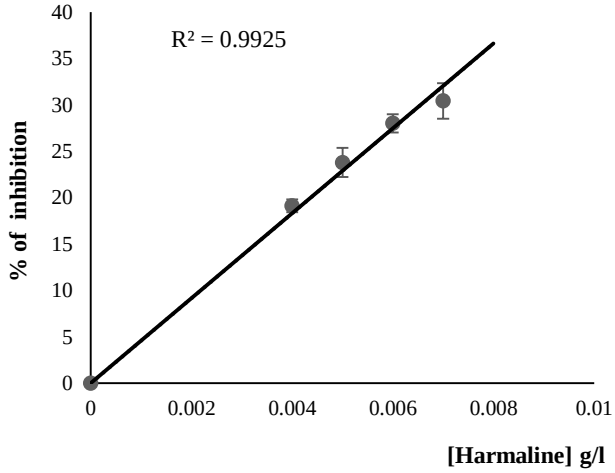


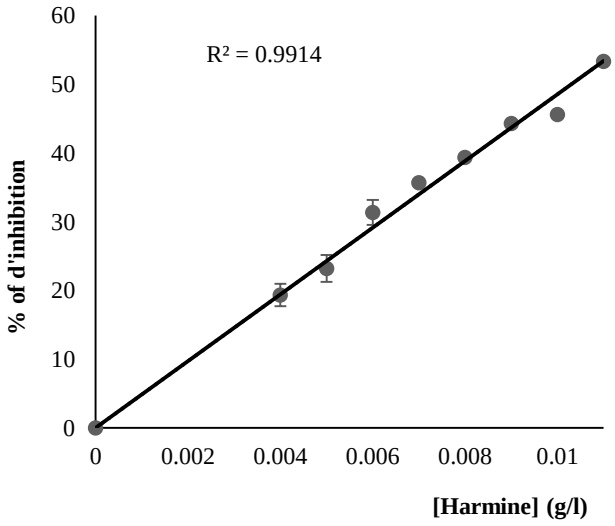
B

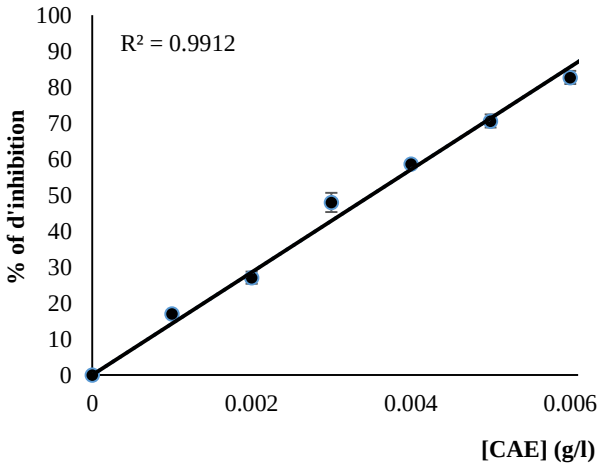


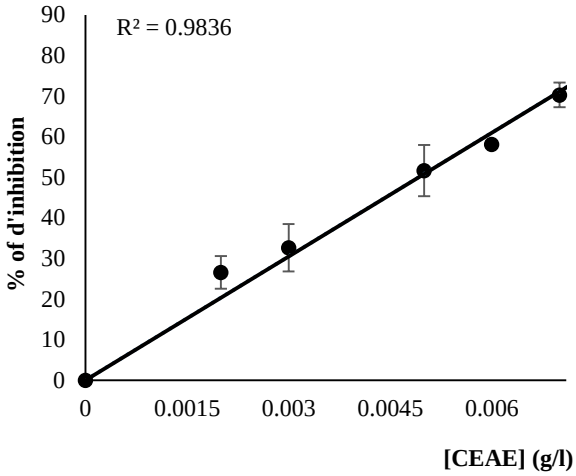
Appendix 04: Inhibition activity plots

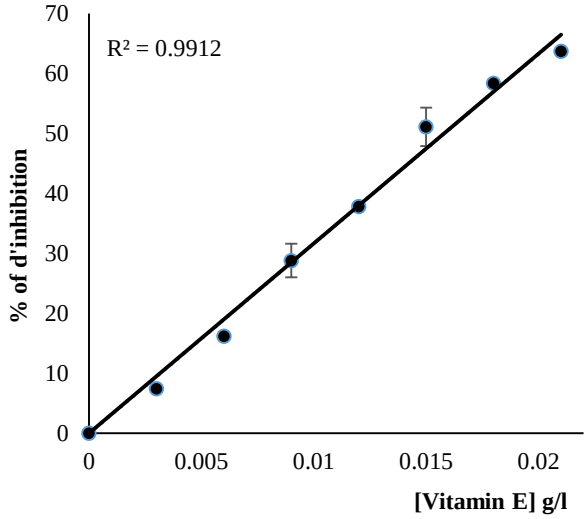


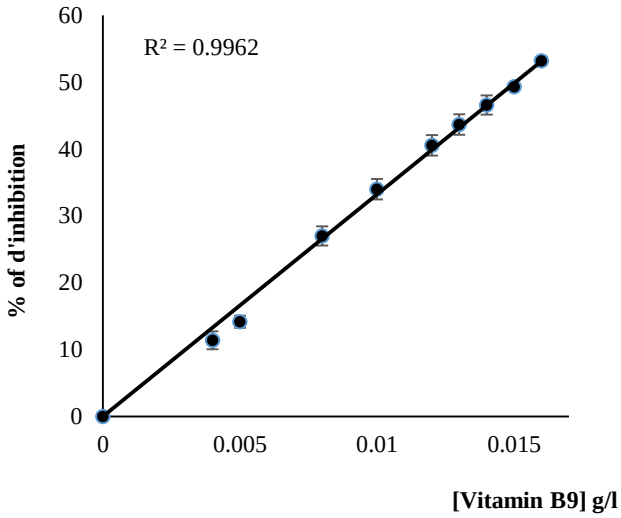


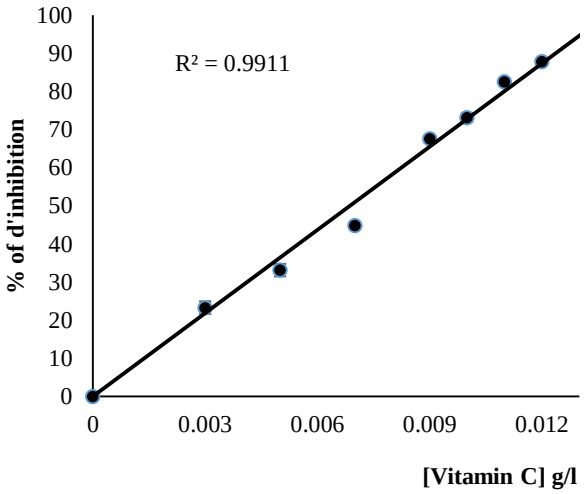


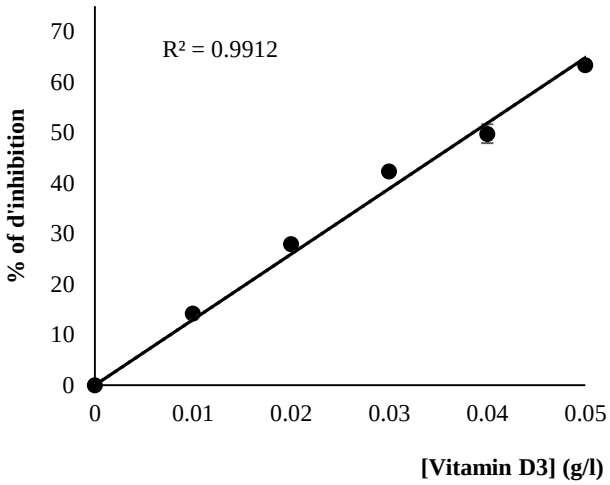

R² = 0.9912
% of d'inhibition
[CAE] (g/l)


R² = 0.9836
% of d'inhibition
[CEAE] (g/l)

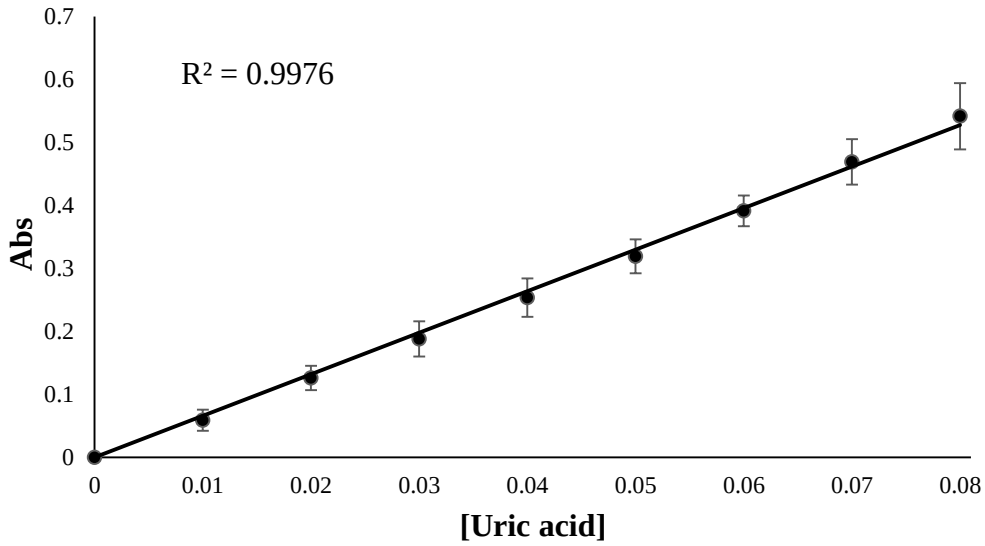

R² = 0.9912
% of d'inhibition
[Vitamin E] g/l


R² = 0.9962
% of d'inhibition
[Vitamin B9] g/l

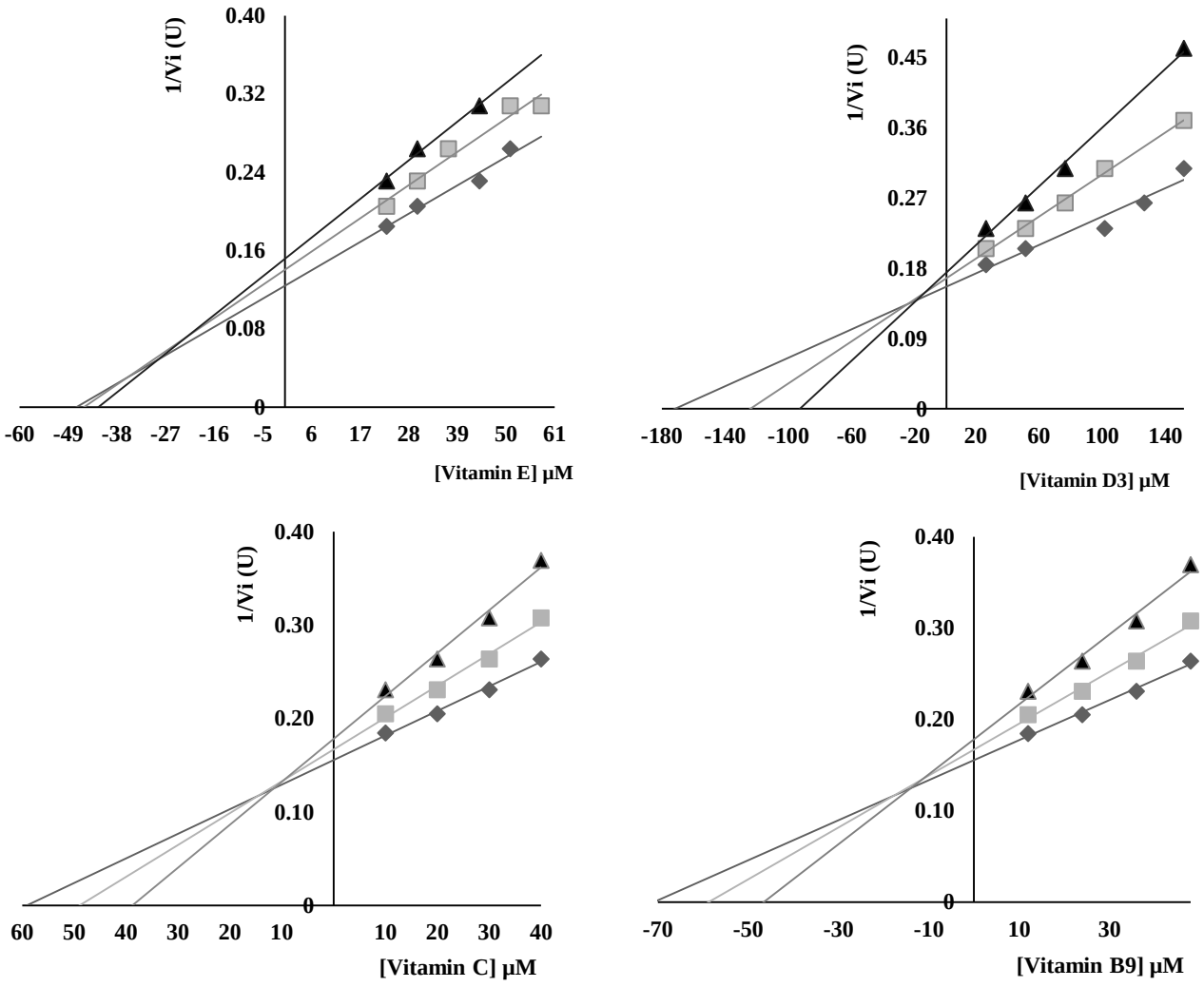

R² = 0.9911
% of d'inhibition
[Vitamin C] g/l

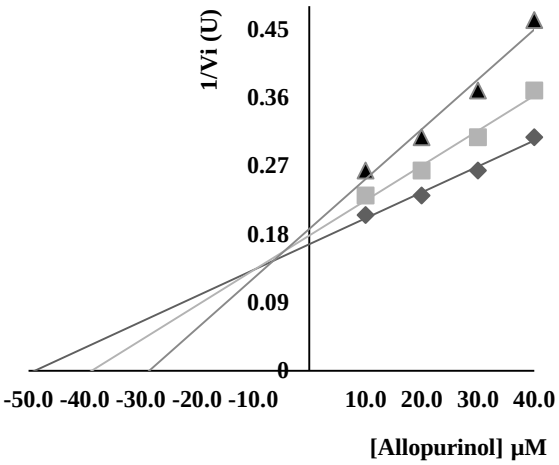

R² = 0.9912
% of d'inhibition
[Vitamin D3] (g/l)

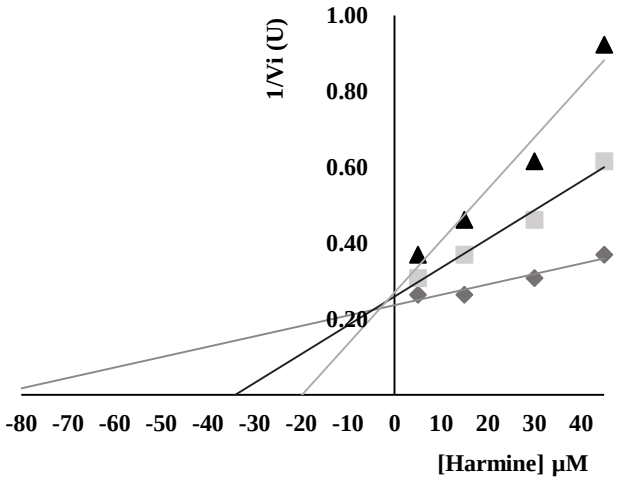
Appendix 05: the uric acid standard curve

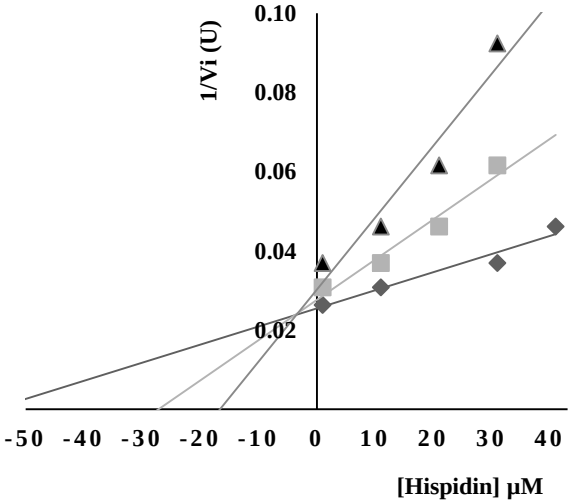


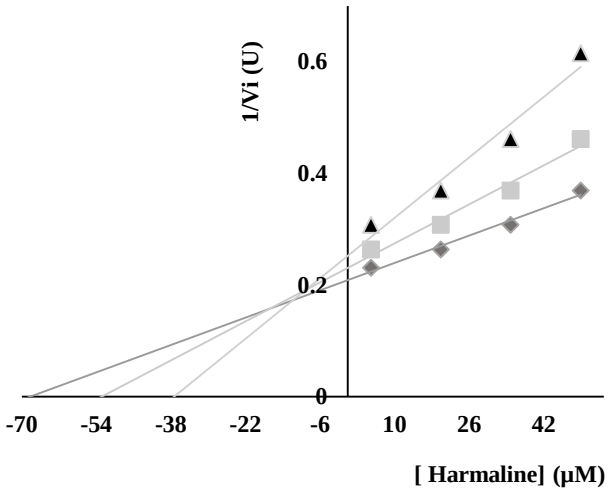
Appendix 06: Inhibitors Dixon plot




1/Vi (U)
0.45
0.36
0.27
0.18
0.09
0
-50.0 -40.0 -30.0 -20.0 -10.0
10.0 20.0 30.0 40.0
[Allopurinol] µM

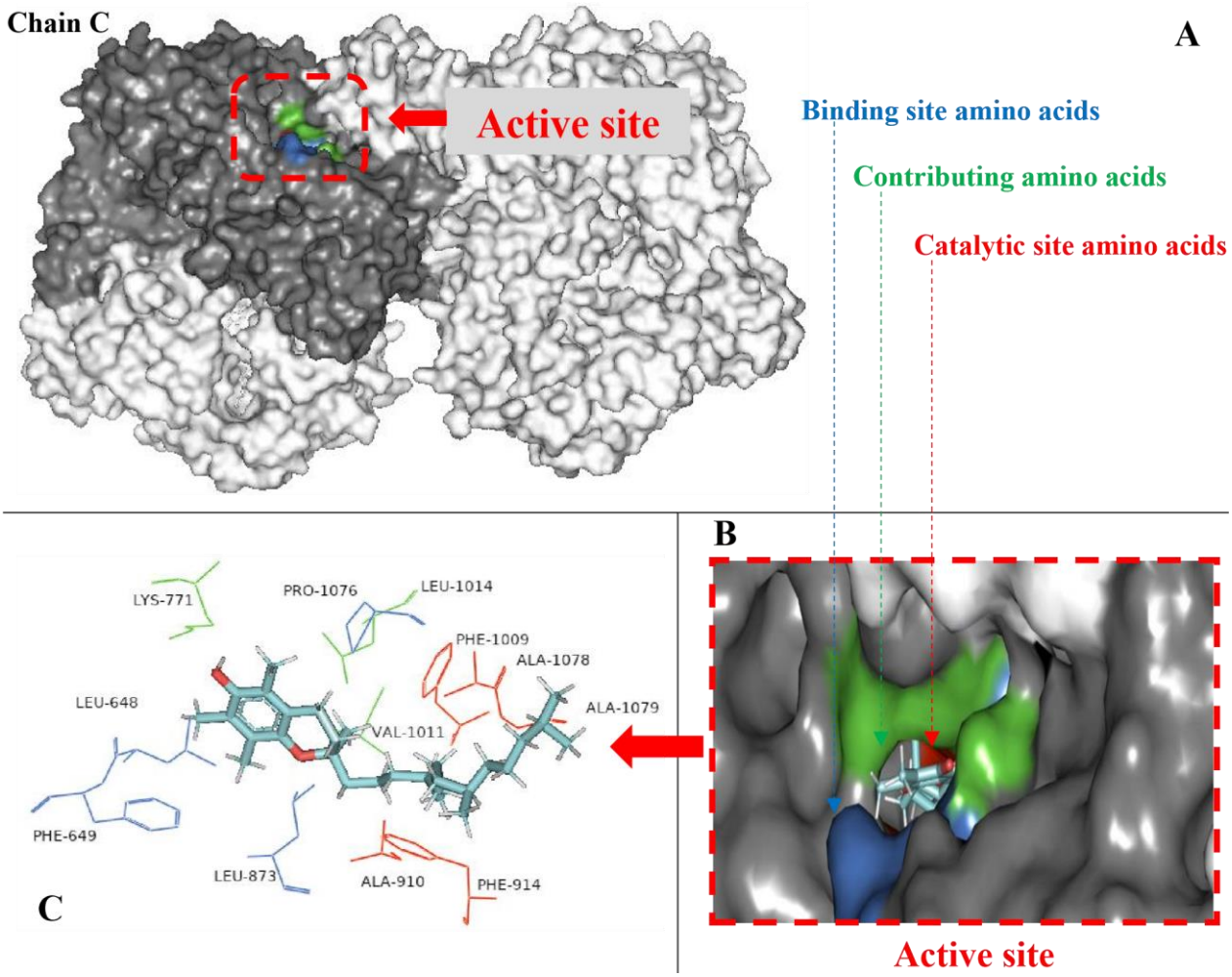

1/Vi (U)
1.00
0.80
0.60
0.40
0.20
-80 -70 -60 -50 -40 -30 -20 -10 0 10 20 30 40
[Harmine] µM


1/Vi (U)
0.10
0.08
0.06
0.04
0.02
-50 -40 -30 -20 -10 0 10 20 30 40
[Hispidin] µM


1/Vi (U)
0.6
0.4
0.2
0
-70 -54 -38 -22 -6 10 26 42
[Harmaline] (µM)

Appendix 07: Example of kinetic assay (Harmine)

Appendix 08: Vitamin E best docking pose in BXO



Appendix 09: Uricase reagent technical sheet

BIOLABO
www.biolabo.fr
MANUFACTURER:
BIOLABO SAS,
Les Hautes Rives
02160, Maizy, France

URIC ACID Uricase method

Reagent for quantitative determination of uric acid in human serum and plasma or urines.

REF 80351	**R1** 6 x 30 mL	**R2** 6 x 30 mL	**R3** 1 x 5 mL
REF 80001	**R1** 2 x 100 mL	**R2** 2 x 100 mL	**R3** 1 x 5 mL
REF 87601	**R1** 6 x 200 mL	**R2** 6 x 200 mL	**R3** 1 x 10 mL

TECHNICAL SUPPORT AND ORDERS
Tel : (33) 03 23 25 15 50
Fax : (33) 03 23 256 256
support@biolabo.fr
Latest revision : www.biolabo.fr

CE

IVD

Made In France

I: corresponds to significant modifications

INTENDED USE

This reagent is designated for professional use in laboratory (manual or automated method).
It allows the quantification of uric acid in human serum and plasma or urines.

GENERALITIES (1) (2)

In humans, uric acid is the major product of the catabolism of the purine nucleosides, adenosine and guanosine.

PRINCIPLE (1) (3)

Uricase acts on uric acid to produce allantoin, carbon dioxide and hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase reacts with a chromogen (amino-antipyrine and dichloro-hydroxybenzene sulfonate) to yield quinoneimine, a red coloured complex.
| The absorbance measured at 505 nm (495-505) is proportional to the amount of uric acid in the specimen.

REAGENTS

R1 URIC ACID Enzymes

Potassium hexacyanoferrate (II)	42	µmol/L
Peroxidase	≥ 450	U/L
Amino-antipyrine	0,150	mmol/L
Uricase	≥ 120	U/L

According to 1272/2008 regulation, this reagent is not classified as dangerous

R2 URIC ACID Buffer

Dichlorohydroxybenzene Sulfonate	2	mmol/L
Tris pH 8.0 at 25°C	50	mmol/L

Preservative
According to 1272/2008 regulation, this reagent is not classified as dangerous

R3 URIC ACID Standard
Uric acid 10 mg/dL (595 µmol/L)

ATTENTION: Flam. Liq.1: H226 Flammable liquid and vapor

P210: Keep away from heat/sparks/open flames/hot surfaces – No smoking, P233: Keep container tightly closed, P280 : Wear protective gloves/protective clothing/eye protection/face protection. P403+235: Store in a well-ventilated place. Keep cool. P501: Dispose of contents/container in accordance with dangerous waste disposal regulations in force in the country

Classification due to: **Ethanol 10 - < 25%**

For more details, see Safety Data Sheet (SDS)

SAFETY CAUTIONS

- Refer to current Material Safety Data Sheet available on request or on www.biolabo.fr
- Verify the integrity of the contents before use.
- Waste disposal: Respect legislation in force in the country.
- All specimens or reagents of biological origin should be handled as potentially infectious. Respect legislation in force in the country.

I Any serious incident that has occurred in connection with the device is notified to the manufacturer and the competent authority of the Member State in which the user and/or patient is based.

REAGENTS PREPARATION

Use a non-sharp instrument to remove aluminium cap.
Add promptly the contents of vial R1 into vial R2.
Mix gently until complete dissolution.
Vial R3: Ready for use

STABILITY AND STORAGE

Stored away from light, well cap in the original vial at 2-8°C, reagents are stable when stored and used as described in the insert:

Unopened,
- Until the expiry date stated on the label of the Kit.

Once opened:
- Reconstitute immediately substrate (vial R1)

Once reconstituted
- Transfer requested quantity and store in the original vial at 2-8°C.
- Working reagent is stable at least 1 month.
- Discard any reagent if cloudy or if absorbance at 505 nm > 0.100.
- Don't use working reagent after expiry date stated on the label.

SPECIMEN COLLECTION AND HANDLING (4)

Unhemolysed serum or plasma (Heparin or EDTA).
Urines: diluted (1+9) in demineralised water before assay.

Uric acid is stable in the specimen for:
- 3 days at room temperature.
- 1 week at 2-8°C.
- 6 months when freeze at – 20°C.

Add NaOH to keep urine alkaline and to prevent uric acid precipitation.

LIMITS (3) (5)

Patient under vitamin C therapy: In order to reduce acid ascorbic interference, let stand specimen 2 hours at room temperature before performing the assay.

For a more comprehensive review of factors affecting this assay refer to the publication of Young D.S.

MATERIAL REQUIRED BUT NOT PROVIDED

1.Basic medical analysis laboratory equipment.
2.Spectrophotometer or Biochemistry Clinical Analyzer

UA_DT_200a_IFU_80351-80001-87601_EN_V01_20201126

QUALITY CONTROL

- REF 95010 EXATROL-N Level I
- REF 95011 EXATROL-P Level II
- External quality control program.

It is recommended to control in the following cases:

- At least once a run.
- At least once within 24 hours.
- When changing vial of reagent.
- After maintenance operations on the instrument.

If control is out of range, apply following actions:

1. Prepare a fresh control serum and repeat the test.
2. If control is still out of range, use a new vial of calibrator or a fresh calibrator and repeat the test.
3. If control is still out of range, repeat the tests with a new vial of reagent.

If control is still out of range, please contact BIOLABO technical support or your local Agent.

EXPECTED VALUES (4)

Serum or plasma	mg/dL	[µmol/L]
Child(*)	2.0-5.5	[119-327]
Men	3.5-7.2	[208-428]
Women(**)	2.6-6.0	[155-357]
Urines	250-750 mg/24h	[1.48-4.43 mmol/24 h]

(*) Higher value in newborn.
(**) Lower during pregnancy.

Each laboratory should establish its own normal ranges for the population that it serves.

PERFORMANCES

On Kenza 240TX, 37°C, 505nm

Detection limit: aprox. 0.03 mg/dL

Precision:

Within run N = 20	*Normal level*	*High level*
Mean mg/dL	3.24	9.05
S.D. mg/dL	0.003	0.007
C.V. %	1%	0.8%

Between run N = 20	*Normal level*	*High level*
Mean mg/dL	6.84	9.4
S.D. mg/dL	0.076	0.172
C.V. %	1.1%	1.8%

On Cobas Mira, 37°C, 505 nm

Measurement interval: between 0.3 mg/dL and 20.0 mg/dL

Comparison study with commercially available reagent:

With n=98 specimens between 2,0 and 200 mg/dL

y = 0,9953 x – 0,025 r = 0,9923

Interferences:

Turbidity	Positive from 0.060 abs
Total bilirubin	Positive interference from 500 µmol/L
Ascorbic acid	Negative interference from 0.5 mg/dL
Hemoglobin	No interference up to 115 µmol/L
Glucose	No interference up to 1010 mg/dL

Other substances may interfere (see § Limits)

CALIBRATION

- REF 95015 Multicalibrator traceable *to SRM 913*
  Or
- Standard (vial R3)

The calibration frequency depends on proper instrument functions and on the preservation of reagent.

Make a new calibration when changing reagent batch, if quality control results are found out of the range and after maintenance operations

PROCEDURE

Manual method

Let stand reagent and specimens at room temperature.

Reagent	1000 µL
Standard / Control or Specimen	25 µL
Mix. Let stands for 5 minutes at 25°C. Record absorbance at 505 (495-505) nm against reagent blank. Colour is stable for 30 minutes.	

Notes:

1. Serum, plasma, or urines diluted (1+9) with demineralised water.
2. Performances with manual procedure should be validated by user.
3. Kenza applications and other applications proposal are available on request.

CALCULATION

Serum or plasma:

$$\text{Result} = \frac{\text{Abs (Assay)}}{\text{Abs (Standard)}} \times \text{Standard concentration}$$

Diluted urines (1+ 9): Multiply the above result by dilution factor 10.

REFERENCES

(1) *TIETZ N.W. Textbook of clinical chemistry, 3rd Ed. C.A. Burtis, E.R. Ashwood, W.B. Saunders (1999) p. 1245-1250.*

(2) *BERNARD S. Biochimie clinique - Instruments et techniques de laboratoire - Diagnostiques médicaux chirurgicaux.2ed éd.1989 p153-156 Ed. MALOINE PARIS.*

(3) *FOSSATI, P., PRENCIPE L., and BERTI G., Use of 3.5-dichloro-2-Hydroxybenzene sulfonic acid / 4 Amino phenazone chromogenic system in direct enzymatic assays of uric acid in serum and urine. Clin. Chem.: 26(227-231) 1980*

(4) *Clinical Guide to Laboratory Test, 4th Ed., N.W. TIETZ (2006) p. 1098-1099.*

(5) *YOUNG D.S., Effect of Drugs on Clinical laboratory Tests, 4th Ed. (1995) p 3-609 to 3-622*

(6) *SRM: Standard Reference Material ®*

Manufacturer	Expiry date	In vitro diagnostic	Storage temperature	Dematerialized water	Biological risk
Product Reference	See Insert	Batch number	Store away from light	Sufficient for	Dilute with