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In vitro and in silico inhibition studies of five essential oils on both enzymes human and bovine xanthine oxidase

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ABSTRACT

In pharmaceutical labs, the major sources of xanthine oxidase enzyme (XO) used for the gout disease experiment are animals and humans. The aim of this study is to find the lucrative source for the experiment by the evaluation in vitro and in silico of the inhibitory activity of five essential oils (EOs), against human and bovine milk xanthine oxidase (HXO and BXO), using molecular docking and an innovative analysis method based on double enzyme detection (DED), investigated in this work for the first time. The DED method proved its efficacy, sensitivity, and quickness. The results show that the five EOs give an important inhibition to BXO and HXO with an IC50 of $3.67 \pm 0.17 \mu\text{g/ml}$, $3.89 \pm 0.11 \mu\text{g/ml}$, $3.76 \pm 0.18 \mu\text{g/ml}$, $2.37 \pm 0.23 \mu\text{g/ml}$, $3.43 \pm 0.56 \mu\text{g/ml}$ for HXO, and to BXO with an IC50 of $6.26 \pm 0.92 \mu\text{g/ml}$, $5.53 \pm 0.82 \mu\text{g/ml}$, $10.59 \pm 1.59 \mu\text{g/ml}$, $1.74 \pm 1 \mu\text{g/ml}$, $5.33 \pm 0.13 \mu\text{g/ml}$, for respectively, cumin (*Cuminum cyminum* L.), fennel (*Foeniculum vulgare* Mill.), coriander (*Coriandrum sativum* L.), anise (*Pimpinella anisum* L.), and caraway (*Carum carvi* L.) EOs. In silico study based on molecular docking using autodock vina program was carried out to study the BXO and HXO inhibition mechanism and involved interactions for the first time. The results show that the five used EOs give an important and similar inhibition effect against XO with both enzyme sources, therefore we propose the bovine milk as a new economic enzyme source for lab experiment, which can promote a new, approach in future gout treatment.

1. Introduction

Aromatherapy based treatment is one of the comparing medications which use essential oils as the genuine, helpful administrators to treat a couple of diseases (Ali et al., 2015). Due to their beneficial antioxidant and anti-inflammatory activities (Zuzarte et al., 2018).

Gout disease continues (Chiang et al., 1994) to spread in the world without any precise treatment with no side effects have been yet discovered until nowadays, it makes the patient exposed, suffering from joints and articulation pains and it may paralyze the movement resulting from the excess accumulation of the uric acid in joints and articulations (Cotelle, 2001; Dalbeth et al., 2009).

The major cause of gout is the overactivity of the xanthine oxidase enzyme (XO), found in the liver (Lin et al., 2016). Its normal function is the oxidation of the hypoxanthine into xanthine and into uric acid (Battelli et al., 2018), (Fig. 1). The expensive cost of lab experiment and

enzyme extraction puts a serious problem to researchers around the world, especially the third world. The most used sources of XO enzyme are human and bovine which are the cases in this paper.

Through this work, we are improving a new economic, lucrative, and effective enzyme source for the XO inhibition future experiment and on the other side searching for a relevant treatment with minimum side effects, using in silico and in vitro studies for the inhibition of bovine xanthine oxidase (BXO) and human xanthine oxidase (HXO) milks. The innovative analysis method used for BXO and HXO inhibition based on the detection of the uric acid by double enzyme technique (DED) is investigated herein for the first time aiming to improve its sensitivity, quickness, and effectiveness. Cumin, fennel, coriander, anise, and caraway are the five essential oils used for the first time as inhibitors to BXO and HXO. To support our study, molecular docking was achieved to illustrate the inhibition mechanism and interaction types with the in vitro model of BXO and HXO, using autodock vina

Fig. 1. Reactions schema catalyzed by xanthine oxidase (XO) enzyme.

with different parameters described here for the first time. The discovery studio visualizer was carried out for results treatment.

2. Materials and methods

2.1. Drugs and chemicals

Human fresh milk was obtained from a volunteer woman 20 days after delivery (weight: 62 kg, age: 34 years and height: 156 cm); her diet was mainly medium in proteins, carbohydrates, and healthy fats. Bovine milk was obtained from a cow farm in the area of Laghouat, Algeria. Uricase and peroxidase enzymes were purchased from Spinreact (Esteve de Bas GIRONA – Spain).

Xanthine substrate, dimethyl sulfoxide (DMSO), K_2HPO_4 , ethylene diamine tetraacetic acid (EDTA) and all other reagents were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH Taufkirchen, Germany). All other chemicals and solvents used were of analytical grade.

2.2. Equipment

Gas chromatograph clarus680 (via Perkin elmer, USA), Fiocchetti Freezer ECT-F -30°C (Via Panagulis, Luzzara, Italy), refrigerated centrifuge from Hettich Rotanta 460R (Bäch SZ, Suisse), Clevenger (via Wisd Wise Therm, GREECE), Microplate reader from URIT UV660 (Guangdong, China).

2.3. Essential oils extraction

Essential oils were isolated from dried and cleaned seed parts of cumin, fennel, coriander, anise, and caraway. The plants were brought from a local market in the area of Laghouat city- Algeria; the different samples of EOs were obtained by hydro-distillation at boiling temperature on a Clevenger device in 3 h. For this purpose, the plants were dried under ambient conditions ($30-38^\circ\text{C}$) for seven days and then 250 g of the seeds were separated for the extraction. The samples were placed in a round flask and then a volume of 1 l of distilled water was added. Subsequently, the obtained EOs were stored after decantation in glass vials with sealed caps at 4°C in the absence of light (pale yellowish essential oil). Volatile oils obtained by hydro-distillation were examined by GC/MS for chemical composition determination.

2.4. Equipment and GC conditions

2.4.1. Sample preparation

A portion of the sample ($2-5\ \mu\text{l}$) was transferred to a GC vial, diluted in hexane ($1-2\ \text{mL}$) then sealed with a high performance septum.

2.4.2. Analytical conditions

Perkin Elmer Gas Chromatograph Clarus680 coupled to Mass

Spectrometer Clarus SQ 8 0 t. The fused-silica Rtx-5MS ($30\ \text{m} \times 0.25\ \text{mmID}$, $0.25\ \mu\text{m}\ \text{df}$, RESTEK, USA) is directly coupled to the mass spectrometer. The carrier gas was helium ($1\ \text{ml/min}$). The program used was 4 min isothermal at 70°C , then 4°C/min to 180°C and 10 min isothermal. The injection port temperature was 250°C and the detector temperature was 280°C . Ionization of the sample components was performed in the EI mode ($70\ \text{eV}$). Mass spectrometer scan range was $30-300\ \text{amu}$. The individual constituents were identified by comparing the mass spectra with spectra stored in the NIST/EPA/NIH mass spectral database. Version 2.0 g, build 19 May 2011.

2.5. Xanthine oxidase enzyme extraction

The extraction process was made according to (Baghiani et al., 2003) in very strict procedures using a refrigerated centrifuge and a fresh human and bovine milk (Sharma et al., 2016). The milk (1 L) was spin-dried in 4500 rpm for 35 min at 4°C and then exposed to several separations steps and various speeds going from 1800 rpm up to 4500 rpm in 4°C .

The floating cream was dissolved in a double volume of phosphate potassium (K_2HPO_4 , 0.2 M) containing 1 mm of EDTA. The mixture was subjected to a soft agitation for two hours, and then spin-dried in 6000 rpm during 20 min in 4°C . Finally, we get enzymes fractions, and then float was filtered, collected and preserved under -30°C for the tests. We decide to work with crude enzyme extracts to accord mimic in vivo conditions when both enzymes are not free or purified but mixed with many proteins.

2.6. Principle of double enzyme detection method (DED)

To find an effective and new way to trace the product produced by HXO and BXO since the uric acid (product target) has no color at the end of the reaction. We proposed the use of specific reagent to detect the uric acid in form of quinoneimine colored in purple (Wu et al., 2015), so we have used the common reagent for the quantitative determination of the uric acid (Spinreact lab) which consists of three bottles. The first is composed by two enzymes, uricase ($120\ \text{U/l}$) and peroxidase ($450\ \text{U/l}$), as well as the hexacyanoferrate (II) of potassium ($42\ \mu\text{mol/L}$) with amino-antipyrin ($0.150\ \text{mmol/L}$) named reagent one. The second is the buffer solution with $\text{pH} = 7.4$ at 25°C ($50\ \text{mmol/L}$) with the dichlorohydroxybenzene sulphonate ($2\ \text{mmol/L}$) named reagent two. The third constitutes the product, which is the uric acid with a concentration of $100\ \text{mg/l}$ ($595\ \mu\text{mol/L}$).

Uricase acts on uric acid produced from both HXO and BXO to produce allantoin, carbon dioxide, and hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase reacts with a chromogen (amino-antipyrine and dichloro-hydroxy benzene sulfonate) to yield quinoneimine, a purple-colored complex. The absorbance then measured at $510\ \text{nm}$ ($490-530$) is proportional to the amount of uric acid in the specimen (Fossati et al., 1980) (Fig. 3).

2.7. Xanthine oxidase inhibition assay

After addition of the enzyme HXO and BXO extract, the inhibitory effect of the five essential oils was tested by following the quantity of the uric acid produced by oxidation of 0.1 g/l of xanthine substrate dissolved in a buffer (KH₂PO₄/K₂HPO₄/KCl, pH 7.4), in the presence of several concentrations of every inhibitor. The final concentration of DMSO in the assay was 25 % in total and used to dissolve the EOs. The absorbance was read at 492 nm in a microplate reader, the inhibitory activity of these essential oils was compared with the two controls allopurinol and febuxostat that are the specific inhibitors of the XO with IC₅₀ of 0.937 µg/ml and IC₅₀ of 0.076 µg/ml, respectively (Kapoor and Saxena, 2014).

The inhibitory activity of EOs was expressed their percentage of inhibition (I%). So:

$$I\% = \left(\frac{AC - AE}{AC} \right) \times 100$$

AC: absorbance in the absence of the inhibitor. AE: absorbance in the presence of the inhibitor.

The IC₅₀ expressed in µg/ml of every essential oil (inhibitory concentration of 50 % of the enzymatic activity) was calculated from the inhibition ratio vs. essential oil concentration curves by regression analysis. All experiments were done at least ten times. The values of standard deviations obtained were mostly less than one.

2.8. Statistical analysis of variance (one way ANOVA)

We used in this paper one-way ANOVA, for result analysis. The value of $p < 0.05$ was considered as statistically significant.

2.9. Molecular docking of the investigated compounds

To improve the sensitivity, effectiveness, and quickness of the double enzyme detection technique (DED) with the new inhibitors of xanthine oxidase. Through this part of this work, we understand the mechanism of HXO and BXO inhibition improved in vitro by the proposed method (DED). For the best knowledge of these interactions and inhibition mechanisms, we should choose the target protein from the protein data bank (PDB) with specific criteria, represented mainly by its 3D active site, which should be interacted with inhibitor. After researching in PDB (protein data bank) (Berman et al., 2000), we found bovine xanthine oxidase with PDB ID: 3NRZ and human xanthine oxidase with PDB ID: 2CKJ. The first thing done is removing all unnecessary ligands as all the water molecules, heteroatoms, any co-crystallized solvent. We selected one major compound in every EOs as an inhibitor for the docking. The 3D inhibitors' structures were obtained from the PubChem database (Kim et al., 2019) according to the Lewis structure. The docking analysis of molecules was carried out using autodock vina program for molecular docking and virtual screening, "molecular docking is a computational procedure that attempts to predict noncovalent binding of macromolecules or, more frequently, of a macromolecule (receptor) and a small molecule (ligand) efficiently, starting with unbound structures, structures obtained from molecular dynamics simulations, or homology modeling. The goal is to predict the bound conformations and the binding affinity" (Trott and Olson, 2010). Autodock vina uses two types of docking; blind docking when we attempt to do docking with no information on the active site, autodock vina finds the site with the most number of accepted conformations, and specific docking when we fix setting manually by the grid coordinates XYZ.

The docking consists of two main programs. Autodock vina program for docking of the ligand (inhibitor) to target receptor (protein) in very specific parameters setting and autodock tools program for the setting of grid box, ligand-receptor setting, generating the pdbqt format file, and hydrogens adding. Autodock tools is the complementary program

came with the main program it consists of four main steps; First step (one), consist of reading our target protein 3NRZ (BXO), and 2CKJ (HXO) and our target inhibitors in the format of pdb file, the pdb files are mostly missing hydrogens; Second step (two), consist of adding the polar hydrogens missing that are required for the pdbqt format, then generate the protein and inhibitors in format of pdbqt file; Third step (three), locating the grid box which is a parameter to define the most adequately center for the docking and will be fixed with the possible best dimensional point named as X,Y,Z especially for every receptor (protein); The fourth step (fourth), selecting torsion count is required to define the rotatable and non-rotatable bond in the ligand (inhibitor). To run docking, we need to provide a text file named as the target receptor – ligand in a separate folder containing ligand and protein information with the set parameters of dimensional point X, Y, Z. Opening the files directory with the command prompt window will run the docking using the autodock vina program and the results will be in the same folder of the file text for the target complex receptor- ligand. The specific docking type was chosen in this work and the lowest interatomic distance modes in Å were selected for analyses. Finally, the docking results generated were directly loaded into the discovery studio visualizer v 4.0 program.

3. Results and discussion

3.1. Essential oils extraction and GC/MS analysis

The average yield of the EOs was ranged from 0.1% to 1.2% all EOs yields and density were calculated (Table 1). Chromatograms (Fig. 5), integrations and compounds detected during analyses are summarized in Table (). Concentrations are based on relative area percentages. As such, results could be semi/quantitative when the volume of the sample is considered. The tentatively identified compounds listed are those which be identified with a high degree of certainty.

We found that the anise essential oil (Table 2, Fig. 5) contained 23 different compounds, including the major compounds anethole or 1-methoxy-4-propenylbenzene (C₁₀-H₁₂-O). His structure containing an aromatic hydrocarbon consisting of a benzene ring substituted by a propenyl group and a methoxy group.

We found in the cumini essential oil (Table 3, Fig. 5) seven different compounds, including the major compounds propanal, or 2-methyl-3-phenyl. His structure containing an aromatic hydrocarbon consisting of a benzene ring substituted by an aldehyde group.

Table 1
xanthine oxidase inhibitory activity with double enzyme detection technique.

	Density	Yield (%)	IC ₅₀ ^d of XO ^e inhibitory activity (µg/ml)	
			HXO ^b	BXO ^c
<i>Carum carvi</i> L.	0.923	0.98	3.43 ± 0.56	5.33 ± 0.13
<i>Pimpinella anisum</i> L.	0.99	0.39	2.37 ± 0.23	1.74 ± 1
<i>Coriandrum sativum</i> L.	0.772	0.1	3.76 ± 0.18	10.59 ± 1.59
<i>Foeniculum vulgare</i> Mill.	0.992	0.37	3.89 ± 0.11	5.53 ± 0.82
<i>Cuminum cyminum</i> L.	0.869	1.2	3.67 ± 0.17	6.26 ± 0.92
Febuxostat ^a	/	/	0.076	
Allopurinol ^a	/	/	0.937	
Human xanthine oxidase (HXO)	/	14.38	/	
Bovine xanthine oxidase (BXO)	/	5.4	/	

^a allopurinol and febuxostat were used as a reference compound in this assay.

^b human xanthine oxidase ^c bovine xanthine oxidase ^d Half inhibitory concentration ^e xanthine oxidase.

Table 2
Chemical composition of the anise essential oil.

Retention Time	Compound Name
7.355	a-pinene
7.856	1,5,5-trimethyl-6-methylene-cyclohexene
8.801	B-pinene
9.156	2,6-octadien-1-ol, 2,7-dimethyl-
9.671	Cyclohexene, 1-methyl-5-(1-methylethenyl) (R)
10.462	Benzene, 1-ethyl-2,4-dimethyl-
10.622	Cyclohexene, 1-methyl-5-(1-methylethenyl) (R)
12.703	1,6-octadien-3-ol, 3,7-dimethyl-
13.328	1,6-octadien-3-ol, 3,7-dimethyl-
16.759	Estragole
17.184	Estragole
21.406	Anethole
22.286	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)-
23.807	2-propanone, 1-(4-methoxyphenyl)-
26.023	Hbenzocycloheptene, 2,4a,5,6,7,8,9,9a-octahydro-3,5,5-trimethyl-9-methylene-, (4aS-cis)-
26.628	4,4-dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo (4.1.0)heptane
27.013	Longifolene-V4)
27.263	1,3-cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl, 1S (Rs)
29.6841	Cyclohexene-1-acrylic acid, 2,6,6-trimethyl-3-oxo-, methyl ester
30.279	Isoaromadendrene epoxide
31.52	Spathulenol
32.135	7-epi-cis-sesquisabinene hydrate
33.506	Retinal

In the caraway essential oil (Table 4, Fig. 5), we determine four compounds with two major compounds, the first one is the D-limonene (C₁₀H₁₆) a terpenic hydrocarbon present in many major oils. The second major compound is the carvone or 2-methyl-5-(1-methylethenyl)-2-cyclohexen-1-one.

We identified in the coriander essential oil (Table 5, Fig. 5), 18 different compounds with one major compound called B-linalool or 3,7-dimethyl-1,6-octadien-3-ol. It is a natural substance, terpenoid liquor that is biosynthesized as D-, L- or dl-linalool by a large group of plants and herbs.

Finally, we found in the fennel essential oil (Table 6, Fig. 5) 14 different compounds, we selected two major compounds the D-limonene and estragole (C₁₀H₁₂O) or 1-allyl-4-methoxybenzene, a colorless liquid with an odor of anise insoluble in water. It is an isomer of anethole, consists of a benzene ring substituted by a methoxy group and an allyl group.

The result achieved from these analyses will be used in silico to understand better the mechanism responsible for the inhibition processes between the major compounds cited and the amino acids in the active site of both HXO and BXO using molecular docking.

3.2. Xanthine oxidase enzyme extraction

The average yield of the enzyme extract obtained from HXO was about two times bigger than BXO enzyme extract (Table 1); we

Table 4
Chemical composition of the caraway essential oil.

Retention Time	Peak Area	Compound Name	CAS#
10.707	1455014784	D-limonene	5989-27-5
14.243	8151198,5	Trans-p-mentha-2,8-dienol	
14.758	23866332	Limonene oxide, cis-	13837-75-7
18.935	1600324992	Carvone	99-49-0

Table 5
Chemical composition of the coriander essential oil.

Retention Time	Peak Area	Compound Name	CAS#
11.777	39437372	Y-terpinene	99-85-4
12.407	14351695	Cis-linalool oxide	5989-33-3
13.503	5094492672	B-linalool	78-70-6
15.214	211471136	(-)-camphor	464-48-2
16.004	231248352	Endo-borneol	507-70-0
16.369	64656976	Terpinen-4-ol	562-74-3
16.879	464275584	a-terpineol	98-55-5
17.064	97599264	Estragole	140-67-0
17.234	61245724	Decanal	112-31-2
18.675	16845762	Propanal, 2-methyl-3-phenyl-	99-49-0
18.85	40301976	Carvone	
19.13	171828832	Geraniol	106-24-1
19.315	42890312	2-decenal, (Z)-	2497-25-8
20.26	88364784	Anethole	104-46-1
20.471	138776752	Thymol	89-83-8
22.601	7604699,5	Phenol, 5-methyl-2-(1-methylethyl)-, acetate	528-79-0
23.532	156963936	Geranyl acetate	105-87-3
26.253	33108028	2-dodecenal	4826-62-4

Table 6
Chemical composition of the fennel essential oil.

Retention Time	Peak Area	Compound Name	CAS#
10.697	89965168	D-limonene	5989-27-5
13.053	72865224	Bicyclo 2.2.1heptan-2-one, 1,3,3-trimethyl	1195-79-5
13.328	18232080	1,6-octadien-3-ol, 3,7-dimethyl-	78-70-6
15.234	10283799	Bicyclo2.2.1heptan-2-one, 1,7,7-trimethyl-, (1S)-	464-48-2
16.024	159996304	Endo-borneol	507-70-0
16.914	394783936	a-terpineol	98-55-5
17.224	4283771648	Estragole	140-67-0
18.7	5673800,5	Benzaldehyde, 4-(1-methylethyl)-	122-03-2
18.865	93496920	(-)-carvone	6485-40-1
20.281	192832368	Anethole	104-46-1
20.486	173414256	Thymol	89-83-8
20.831	12114243	Phenol, 2-methyl-5-(1-methylethyl)-	499-75-2
22.601	10159122	Phenol, 5-methyl-2-(1-methylethyl) acetate	528-79-0
29.674	36951968	3-methoxycinnamaldehyde	56578-36-0

concluded that the enzyme richness in the human kind is bigger comparing to bovines. The enzyme activities were tested by the proposed method of (DED) using *Uricase POD* liquid, after incubating the enzyme

Table 3
Chemical composition of the cumin essential oil.

Retention Time	Peak Area	Compound Name	CAS#
8.876	93359424	B-pinene	127-91-3
10.502	460181760	Benzene, 1-methyl-3-(1-methylethyl)	535-77-3
15.194	46616592	Bicyclo2.2.1heptan-2-one, 1,7,7-trimethyl-, (1S)-	464-48-2
16.934	98980304	1,3-cyclohexadiene-1-methanol, 4-(1-methylethyl)	1413-55-4
18.16	56692320	Phenol, 3(1-methylethyl)-	618-45-1
18.69	889608512	Propanal, 2-methyl-3-phenyl	
21.561	124283960	Cyclohexanbutanal, 2-methyl-3-oxo-, cis-	92485-93-3

Fig. 2. A: the 3D structure of the human xanthine oxidase HXO 2CKJ (Chain A) colored by structure; B: the 3D structure of the bovine xanthine oxidase 3NRZ (Chain C) colored by structure; C: zooming in the active site of both enzymes complexed with ligand: flavin-adenine dinucleotide (FAD).

Fig. 3. Schema of reactions reported from literature (Avis et al., 1956).

(a) Incubation of uric acid with urate oxidase [only the final products are reported (hydroxyisouric acid; the product of the enzyme reaction is unstable and not enzymatically converted into allantoin, CO_2 , and H_2O_2)], (b) DCHBS oxidation catalyzed by HRP in the presence of hydrogen peroxide, and (c) phenoxyl radical reaction with 4AAP, producing a purple quinoneimine dye.

extract with 0.1 g/l of xanthine substrate diluted in buffer at pH of 7 for 30 min, then read at 492 nm after adding *Uricase* -*POD*. The results showed the presence of uric acid colored in purple after reacting with the *Uricase* *POD*. Liquid according to (Fossati et al., 1980), and that control and confirm the activity of our enzyme extract.

3.3. Xanthine oxidase inhibition assay with the DED method

It was observed that the changes ranged from one to three times bigger as max between the inhibitions values in the human milk and bovine milk enzymes (Table 1). The EOs in the HXO achieved less IC₅₀. We conclude that the EOs had an affinity toward the HXO enzyme compared with the bovine milk enzyme (BXO), this explains the nature of both source origins and its effect on the genetic structure compared with humans. Although this genetic structure changing did not affect very significantly on the inhibition between the two sources.

Experiments on XO inhibition were conducted from 1999 to nowadays by different groups of researchers using two methods, spectrophotometric method measuring the released amount of uric acid at 292 nm (Kong et al., 2000) and HPLC method measuring the same product (Mohamed Isa et al., 2018). The first method is less sensitive when we use inhibitors with aromatic rings because they absorb at the same wavelength then interfering with the results. The second method is effective and precise, but it takes a long time. Hence, this paper proposes a new approach to measure uric acid based on the use of two enzymes instead of one enzyme (in the routine method), which increases the accuracy of the detection of the product by the reagent which is the enzyme 2 which is originally the specific reagent of the product in our case (uric acid). According to the results obtained by statistical analysis of variance indicate that all groups tested have a *F* stat bigger than the *F*_{crit} and all of them have *P*-value smaller than 0.05 briefly, our proposed method is good. It has many interesting, attractive

Fig. 4. The docking pose with the inhibitors on the active site of HXO and BXO for A: anethole, B: carvone, C: d-limonene, D: linalool, and E: propanal. Inhibitors: presented in sticks and colored by default atom color, amino acids: presented in lines and colored by default atom color.

and valuable applications in drug design with practical quickness. The obtained results show that all the essential oils inhibit the activity of the HXO and BXO very significantly, compared with the controls

(allopurinol and febuxostat) (Table 1). Used as an effective medicine for this disease, which causes serious side effects. We could not discuss our results with other researches in literature because there is no research

Fig. 5. Gas chromatography (GC) profiles of EOs from five species: (AG) anise essential oil; (CC1) cumin essential oil; (CC2) caraway essential oil; (CS) coriander essential oil and (FV2) fennel essential oil.

similar to ours, only the nearest one was done by a group of Taiwan researchers when attempting to inhibit the XO by the essential oil of *C. osmophloeum* leaves and presented the strongest XO inhibition activity with an IC₅₀ of 16.3 mg/ml (Wang et al., 2008).

3.4. Molecular modeling and docking of the investigated compounds

The analyses using the discovery studio visualizer program revealed that the human xanthine oxidase (HXO) with PDB ID: 2CKJ (Fig. 2) is an enzyme with four chains of 5314 amino acids named A: (ALA3-TRP1329), B: (THR2- ARG1332), C: (ALA3- ARG1332), and D: (ALA3-ARG1332). It is a big size enzyme with a molecular weight of 592 kDa. The active site is a cavity divided into two sections. One section imparts specificity to the ligand (substrate or inhibitor), and the second is preserved to the cofactors, in this case, is complex with phosphate ions (PO₄³⁻), Flavin-adenine dinucleotide (FAD), glycerol (GOL), acetic acid (ACY), and iron²⁺ sulfide (FES). The docking site was centered at the following amino acids of the chain A: (GLN112, CYS150, GLN768, GLY797, GLY798, GLY1262, PHE799, PHE915, GLY800, ARG913, MET1039, GLY1040, GLN1041, ALA911, SER1081, GLN1195, and GLU1262). The bovine xanthine oxidase (BXO) with PDB ID: 3NRZ (Fig. 2) is an enzyme with three chains of 1222 amino acids named A: (THR2- LYS165), B: (PRO224- GLY528), and C: (ASP571- LYS1326). It is an enzyme with a molecular weight of 274 kDa. The active site of 3NRZ is a small cavity, and from a structural point of view, it is divided into two sections. One section impart specificity to the ligand (substrate or inhibitor), and the second is preserved to the cofactors, in this case is complex with hypoxanthine (HPA), flavin adenine dinucleotide (FAD), iron²⁺ sulfide (FES), dioxothiomolybdenum (MOS), phosphonic acid-mono-(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). The docking site was centered at the following amino acids of the chain C: (GLU802, MET1038, LEU873, SER876, ARG880, ALA910, GLY913, PHE914, PHE1005, SER1008, PHE1009, THR1083, THR1010, VAL1011, LEU1014, PRO1076, ALA1078, ALA1079, SER1082, and GLU1261). The docking parameters were fixed on the grid box with (x = 37.338, y = 19.791, z = 17.854) and (x = 79.017, y = 77.284, z = 141.85) for respectively 3NRZ (BXO) and 2CKJ (HXO), hydrogens were added to both proteins. The docking results showed multiples solutions with up to 50 confirmations saved per molecule. The lowest interatomic distance modes in Å between ligand-receptor were used (Fig. 4). In addition, we defined the amino acids in the binding site of both HXO and BXO responsible for the interaction with the inhibitors, CYS150, MET1039, ARG913 GLN768, and PHE799 are responsible for the interaction in the active site of HXO, While THR1010, PHE1009, PHE914, ALA1079, LEU873, SER1082 are responsible for the interaction in the active site of BXO. The results show that the anethole has a minimum distance of 3.24 Å with THR1010 in the BXO, and 3.67 Å with CYS150 in the HXO. For carvone, we scored 3.32 Å with ALA1079 in the BXO, and 4.21 Å with CYS150 in the HXO. With D-limonene, we saved 3.69 Å with PHE914 in the BXO and 4.21 with CYS150 in the HXO. While in the linalool, 3.12 Å with THR1083 in the BXO and 2.96 Å with GLN768 in the HXO. In addition, we saved in propanal 3.32 Å with THR1010 in the BXO and 3.25 Å with PHE799 in the HXO. The all-interatomic distances saved was ranged from 2.96 Å to 4.21 Å with a difference of only 1.25 Å, in this case we concluded that all the inhibitors tested in both HXO and BXO are the same in term of interatomic distance. The types of involved interaction were determined, where we observed in this context that the bonds type between the CYS, GLN, and PHE in the HXO and the different inhibitors reported in this work were hydrogens bonds type, while THR, ALA, PHE, and SER active site amino acids in the BXO were also hydrogens bonds type. For the first time in this study, the docking of HXO and BXO with these parameters is achieved and no further studies were found in this context.

3.5. In silico and in vitro assays

In this study, we have shown the results obtained by inhibition of the XO responsible for gout disease (Table 1) by the power of five EOs. The enzyme has been extracted from two different sources: human milk and bovine milk, which are two of the most frequent, used sources in lab experiments, to compare the genetic effect on the enzymes active site structure and their affinity toward this EOs and to understand and clarify the differences between them. We concluded that the differences between the two xanthine oxidase enzyme from human and bovine are not significant in term of inhibition affinity, interatomic distance and bond type. After the results achieved in this research, it is possible to carry out future laboratory experiments to develop a drug for this disease using available XO enzyme from bovine milk instead of human milk, which is very difficult and charged. We also use in this work an innovative method to detect the colorless product (uric acid) in the original enzyme method, which is characterized by its effectiveness, ease, and economical compared with the methods used in the other research.

4. Conclusion

Through this work, we tested the most known essential oils in the area of Laghouat city, Algeria for their activity to inhibit the xanthine oxidase enzyme from two different sources, which is a therapeutic target for the gout disease. Based on the achieved results, we suggest the use of the XO enzyme extracted from bovine milk as an alternative to future laboratory experiments for ease of access, adequate, and lower costs. The evidence from this study suggests that the method of double enzyme detection (DED) is accurate, precise, quick, sensible, reproducible, and efficacy method according to ANOVA analysis.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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