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Amperometric biosensor based on Prussian Blue and Nafion modified screen-printed electrode for screening of potential xanthine oxidase inhibitors from medicinal plants

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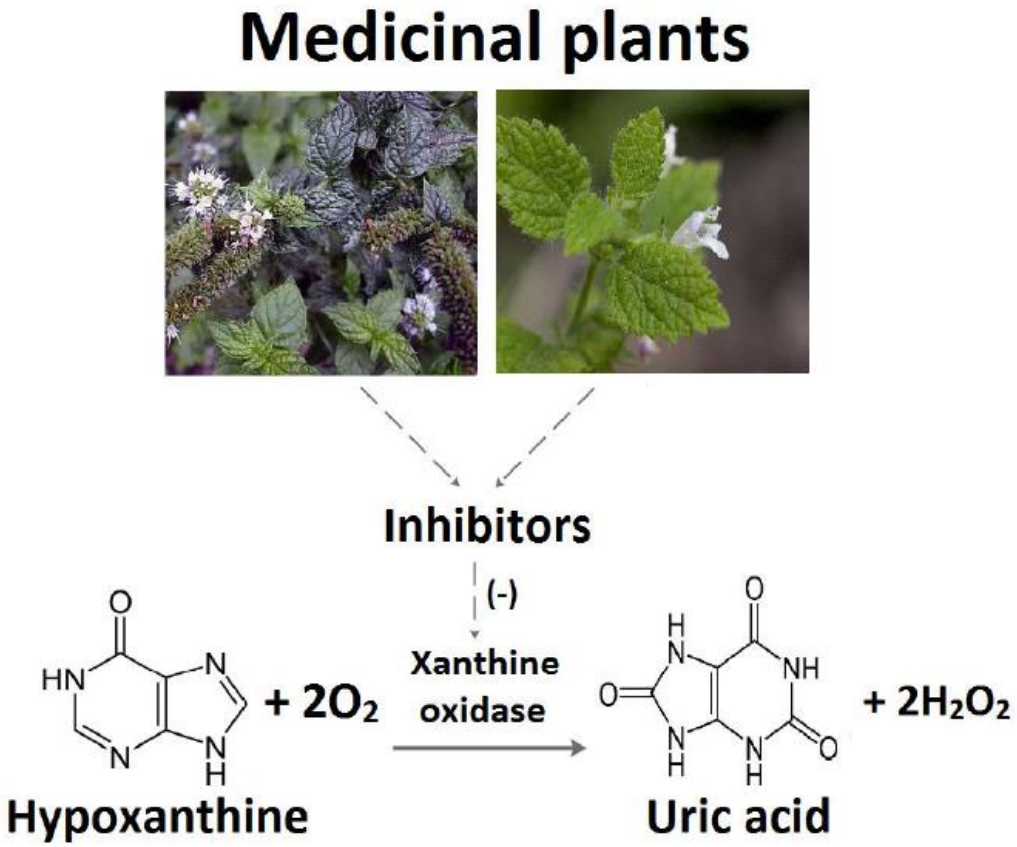
Graphical Abstract



Highlights

- ✓ The development of a simple and sensitive amperometric biosensor for the screening of potential xanthine oxidase inhibitors from medicinal plants
- ✓ Comparison of the biosensor method with the standard spectrophotometric method
- ✓ Application of the proposed inhibitive biosensor for screening of plant extract as new drugs.

Abstract

A simple and sensitive amperometric biosensor was developed for the screening of potential xanthine oxidase inhibitors from medicinal plants. This biosensor was prepared by immobilization of xanthine oxidase on the surface of Prussian Blue modified screen-printed electrodes using Nafion and glutaraldehyde.

The developed biosensor showed a linear amperometric response at an applied potential of +0.05 V towards the detection of hypoxanthine from 5 µM to 45 µM with a detection limit of 0.4 µM (S/N=3) and its sensitivity was found to be 600 $mA.M^{-1}.cm^{-2}$. In addition, the biosensor exhibited a good storage stability.

The inhibition of xanthine oxidase by allopurinol was studied under the optimized conditions. The linear range of allopurinol concentration is obtained up to 2.5 µM with an estimated 50% of inhibition $I_{50} = 1.8$ µM.

The developed biosensor was successfully applied to the screening of xanthine oxidase inhibitors from 13 medicinal plants belonging to different families. Indeed, Moroccan people traditionally use these plants as infusion for the treatment of gout and its related symptoms. For this purpose, water extracts obtained from the infusion of these plants were used for the experiments. In this work, 13 extracts were assayed and several of them demonstrated xanthine oxidase inhibitory effect, with an inhibition greater than 50% compared to spectrophotometry measurements that only few extracts showed an inhibition greater than 50%.

Keywords: Prussian Blue; Screen-printed electrode; Amperometric biosensor; Xanthine oxidase; Allopurinol; Medicinal plants

1. Introduction

Xanthine oxidase (XO) (EC 1.2.3.2) catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid and H_2O_2, the final reactions of the metabolism of purine bases. The overproduction and/or underexcretion of this acid lead to the incidence of hyperuricemia such as gout [1,2]. Accordingly, the use of XO inhibitors is one of the therapeutic approaches to treat gout by blocking the production of uric acid. Allopurinol is one of the XO inhibitors under the clinical application used to treat gout, but this drug suffers from many side effects such as hepatitis, nephropathy, allergic reactions, etc. [3-5]. Therefore, there is an urgent need to search for new XO inhibitors with increased therapeutic activity and less side effects.

Natural products are excellent sources of new compounds in search for new medicines for the treatment of diseases. Morocco, a country possessing a long history of traditional medicinal system, has a number of medicinal plants used for gout, but no systematic investigations have been reported until now. In the present study, thirteen medicinal plants were selected to screen their XO inhibitory activity based on their ethnomedical use in the treatment of gout.

Nowadays, accurate, rapid, cheap and selective methods are needed for use in clinical diagnostic and food safety. The electrochemical biosensors have shown advantages like simplicity, rapid, high sensitivity, specificity, low cost and being economically viable.

Until now, a variety of amperometric biosensors for xanthine/hypoxanthine detection, based on the electro oxidation of hydrogen peroxide formed [6-10] have been reported. However, direct hydrogen peroxide amperometric detection at conventional electrodes is only possible at a potential of. +0.6V versus Ag/AgCl. At this high potential, the presence of easily oxidisable compounds such as ascorbate, phenols, urate, etc. can interfere in the measurement, being oxidized at the electrode together with the hydrogen peroxide [11]. To overcome this problem, the use of mediators such as Prussian Blue (PB), which were able to reduce the enzymatic product, hydrogen peroxide, is proposed. This PB mediator may lead to a high selectivity owing to the low operating potential.

The screen-printed electrodes are frequently used in analytical applications because of their unique properties such as mass production, low cost, high reproducibility, small size, etc. [12]. Those electrodes modified with PB are an excellent substrate for the development of oxidase enzyme biosensor [11,13-15]. In fact, these biosensors can represent a good alternative method to spectrophotometry. Indeed, this is the first report of the use of biosensor to evaluate the enzyme inhibitors from medicinal plants.

In this paper, we report an amperometric biosensor of XO, which was immobilized onto the surface of the SPEs modified with PB. To prevent loss of the enzyme molecules and to improve the stability of the biosensor, Nafion (polymer) was used.

Indeed, the purpose of this study was to develop a biosensor incorporating immobilized xanthine oxidase and Prussian Blue to evaluate XO inhibition from different medicinal plants so as to discover a natural substitute of plant origin that can be used as an alternative to allopurinol for the treatment of gout. The optimum working conditions as the substrate concentration, the method of immobilization and the pH were investigated.

2. Experimental

2.1. Materials and reagents

XOD (EC 1.17.3.2, 0.1 units/mg protein, Grade I from bovine milk) allopurinol and hypoxanthine were purchased from sigma (USA). Glutaraldehyde was supplied by Shanghai Lingfeng Chemical Reagent Co., Ltd. All the other chemical reagents used were of analytical grade and without further purification. Phosphate buffer saline (PBS, 0.05 M) with various pH values was prepared by mixing K2HPO4 and KH2PO4, containing 0.1 M KCl. All the solutions were prepared with distillated water.

2.2. Apparatus and measurements

The Amperometric and voltammetry measurements were carried out at room temperature using a PalmSens potentiostat interfaced to a computer. The measurements were performed by the three-electrode system. The polyester film was used as substrate for printing the graphite-working, graphite-counter and Ag

reference electrodes [16]. In all measurements 0.05 M PBS + 0.1 M KCl was used as an electrolyte.

Screen-printed electrodes (SPEs) consisted of a working electrode in graphite, a reference electrode in silver/silver chloride, and a counter electrode in graphite (Ø= 3 mm). The electrodes were printed with a 245 DEK (Weymouth, UK) screen-printing machine. Graphite based ink (Electrodag 421, Acheson, Henkel, UK), silver/silver chloride ink (Electrodag 4038 SS) and insulating ink (Carboflex 25.101.S, Acheson, Henkel, UK) were used. SPEs were produced in the laboratory of University of Tor Vergata of Rome (Italy).

For the spectrophotometric measurements, a JENWAY 6850 UV–vis spectrophotometer was used.

2.3. Plant materials

Thirteen medicinal plants were collected from Casablanca region in the west north of Morocco. Plant parts have been chosen in relation to Moroccan popular medicine use: The leaves and flowers of twelve plants: *Ailanthus altissima (Simaroubaceae), Artemisia herba-alba (Asteraceae), Caryophyllus aromaticus (L.) (Myrtaceae), Ginkgo biloba (L.) (Ginkgoaceae), Hyssopus officinalis (L.) (Lamiaceae), Lavandula angustifolia (Lamiaceae), Melissa officinalis (L.) (Lamiaceae), Mentha spicata (L.) (Lamiaceae), Rosmarinus officinalis (L.) (Lamiaceae), Solidago (L.) (Asteraceae), Thymus vulgaris (L.) (Lamiaceae)* and *Urtica dioica (L.), (Urticaceae),* and the stigma of *Zea mays (L.) (Poaceae).* These plants were extracts and analyzed on XO inhibitory properties.

2.4. Preparation of extracts

The method of extraction of the selected plants was chosen according to the traditional use of these plants to treat gout. The different parts of each medicinal plant were air-dried. The dried plant materials were cut into small particle size. Two grams of each of the dried plant materiel were added into 20 mL boiled water, and incubated for 30 min. Each extract was filtrated using whatman No. 1 filter paper. Then, it was subjected to XO inhibitory activity assay spectrophotometrically at 290 nm and electrochemically to determine the XO inhibitory properties.

2.5. Preparation of the modified Prussian Blue film SPE electrode

2.5.1. Electrode pre-treatment

Prior to Prussian Blue modification screen-printed electrodes (SPEs) were pretreated in PBS solution (0.05 M, pH 7.5) containing 0.1 M KCl, by applying an anodic polarization potential of +1.7 V for 3 minutes [15].

2.5.2. Chemical deposition of Prussian Blue (PB)

The SPEs were then modified with Prussian Blue and this was accomplished by preparing a "precursor solution" and placing a drop (10 μL total volume) on the working electrode area [16]. This solution was obtained by mixing 5 μL of 0.1 M potassium ferrocyanide in 10 mM HCl with 5 μL of 0.1 M ferric chloride in 10 mM HCl directly on the surface of the working electrode.

The drop was placed carefully and exclusively on the working electrode area, in order to avoid any internal resistance on the system. The solution was incubated on the electrode for 10 min, and then rinsed with a few milliliters of 10 mM HCl. The electrodes were then left 90 min in the oven at 100 °C to obtain a more stable and active layer of PB [15]. The PB-modified electrodes were stored dry at room temperature in the darkness.

2.6. Enzyme Immobilization

Xanthine oxidase (XO) was immobilized into PB modified SPEs using two step cross-linking method. A drop of 5 μL of glutaraldehyde solution was applied exclusively on the working electrode. The solution was then left to dry at room temperature for 1h. Then 7 μL of a mixture of BSA, enzyme and Nafion was applied on the working electrode. The mixture was prepared by mixing 5 μL of BSA (2%), 5 μL of Nafion (2%) and 20 μL of xanthine oxidase (5 U/mL). Once the solution had dried at room temperature and washed several times with buffer solution (pH 7.5 0.05 M phosphate buffer), the biosensor was stored in phosphate buffer at 4°C when not used.

2.7. Biosensor response measurements

The inhibitory action of plant extract on the enzymatic activity of XO was followed by consideration of the electrocatalytic reduction by PB-membrane modified

electrode of the enzymatically formed H_2O_2 with an applied potential of +0.05 V versus internal reference.

A well known XO inhibitor, allopurinol was used as a positive control for the inhibition test. The addition of allopurinol causes inhibition of enzyme, consequently decreasing the amount of liberated enzymatic reaction products.

The PB-SPE/XO/Nafion modified electrodes were immersed into a cell containing 10 mL of phosphate buffer solution 0.05 M + KCl 0.1 M, pH 7.5. The solution was stirred continuously in the open air with a magnetic stirrer to maintain a constant oxygen concentration in the solution. When a baseline was establish, 40 µM of hypoxanthine (substrate) was spiked to record a steady-state current (I_0) before adding inhibitor. The concentration of added plant extract was increased stepwise, by adding defined volumes of an appropriately diluted solution to inhibit the enzyme activity, causing a decrease in current (I_1). The degree of inhibition (I(%)) due to the inhibitor effect of the plant extract was evaluated according to the equation:

$$I(\%) = \frac{I_0 - I_1}{I_0} \times 100$$

Where I_0 and I_1 are the current recorded before and after inhibition respectively.

2.8. Assay of xanthine oxidase inhibitory activity

The inhibitory effect on XO was measured spectrophotometrically at 290 nm under aerobic condition, with some modification, following the method of Unno et al. (2004) and Umamaheswari et al. (2007). The reaction mixture consisted of 200 µL of 0.05 M sodium phosphate buffer (pH 7.5), 400 µL of samples extract with distilled water and 100 µL of freshly prepared enzyme solution (0.2 U/mL of xanthine oxidase in phosphate buffer). The assay mixture was pre-incubated at 37°C for 15 min. Then, 200 µL of substrate solution (0.1 M of hypoxanthine) was added into the mixture. The absorbance was measured using UV/VIS spectrophotometer against a blank prepared in the same way but the enzyme solution was replaced by the phosphate buffer.

The equation reported by Amine et al (2014) was used to evaluate the degree of inhibition of XO activity. Thus, the degree of inhibition was calculated using Eq. 1,

with V_0 is the initial velocity in absence of inhibitor (without test extract) and V_i is the initial velocity in presence of inhibitor (with test extract).

$$y = \frac{V_O - V_i}{V_O} \times 100 \qquad (1)$$

3. Results and Discussion

3.1. Cyclic voltammetry characterization in the presence of H_2O_2

The modified SPEs electrodes with Prussian Blue film were characterized by cyclic voltammetry in the absence and presence of hydrogen peroxide. The cyclic voltammograms of the non-enzymatic electrode SPE/PB measured without and with the addition of 4 mM H_2O_2 in 0.05 M phosphate buffer, pH 7.5 containing 0.1 M KCl was done. The redox couple, corresponding to PB oxidation/reduction is observed in the absence of H_2O_2. When hydrogen peroxide is added, its reduction starts at +0.07 V and oxidation around +0.1 V; an enhancement of the PB reduction peak current is also observed.

This observation illustrate that PB play a significant role, and this effect can be used to enhance the current generated and to overcome the problem of reducing the electrochemical interference by working at potentials close to 0.0 V.

The catalytic mechanism of XO can be expressed by the following equations:

$$H_2O_2 + PB_{red} \longrightarrow PB_{ox} + 2OH^-$$

$$PB_{ox} + 2e^- \longrightarrow PB_{red}$$

3.2. Optimization of experimental parameters for amperometric detection

In this work, an amperometric biosensor of XO was prepared. Therefore, the experimental parameters that can affect the performance of the biosensor, namely the enzyme immobilization method, pH of the supporting electrolyte and hypoxanthine concentration were investigated in order to optimize the biosensor performance.

3.2.1. The effect of the immobilized method

The main idea was to develop a simple and sensitive biosensor. For that purpose, the choice of the method of immobilization is very important. Based on previous works where different immobilization methods were compared [9,16,18-21], xanthine oxidase was chosen to be immobilized by cross-linking method with two steps using glutaraldehyde and bovine serum albumin. In order to facilitate the adherence of the enzymatic membrane on electrode surface and to increase the stability of biosensor [15], the enzyme was mixed with Nafion polymer (Nafion/XOD). Normally, Nafion is used due to its film hydrophobicity and enzyme-favored environment, it is also used in order to enhance selectivity of the biosensor by electrostatic repulsion of unwanted species [19]. The amount of immobilized enzyme was varied in order to evaluate the best response towards hypoxanthine, allopurinol and plant extracts. Different loadings were used: 0.1 and 0.5, 1.0, 2.0 mg mL^{-1}. The response toward substrate hypoxanthine increases with increasing the enzyme concentration; however, the response to inhibitor gradually decreases with increase of enzyme loading from 0.5 to 2.0 mg mL^{-1}. As compromise, an enzyme concentration of 1.0 mg mL^{-1} was selected for use in further experiments.

3.2.2. The effect of Hypoxanthine concentration

The choice of the appropriate substrate concentration can influence the degree of inhibition. Accordingly, for the inhibition study the amount of substrate has to be carefully adjusted [18]. The effect of substrate (hypoxanthine) concentration on the inhibition of xanthine oxidase by plant extracts and allopurinol was thus examined. When the concentration of HX was lower than 20 µM, the current response generated by the biosensor was as small as the inhibition by plant extracts and allopurinol could not be seen. However, in the case of competitive inhibition, at too high substrate concentration (>100 µM), all active sites of XO enzyme will be occupied by the substrate and it will be insensitive to inhibition by allopurinol (competitive inhibitor). Therefore, a concentration of 40 µM HX was selected as the fixed concentration for further allopurinol and plant extracts amperometric measurements.

3.2.3. The effect of pH

The response of the enzyme depends significantly on the pH of the medium, because each enzyme has an optimum pH in its natural environment. Since the pH can affect the protonation and deprotonation of the amino acids of the enzyme active site, the affinity of the enzyme to the substrate can be changed accordingly. Therefore, the effect of the pH on the electrochemical behavior of the PB-SPE/XO/Nafion modified electrode was investigated over the pH range between 6.5-8.5 in 0.05 M PBS (containing 0.1 M KCl) in the presence of 40 µM hypoxanthine in order to obtain efficient response of this biosensor. As it can be seen from Fig. 1, the response of the enzyme biosensor increases with the increase of pH from 6.5 to 7.5 and then decreases. Thus, the optimum response current is reached at about pH 7.5.

3.3. Analytical performance of the developed biosensor

The biosensor developed in this work, use xanthine oxidase, which catalyze the production of H_2O_2, which can be detected electrochemically using PB redox mediator [22]. In consideration of the prepared electrode with XO, the biocatalytical process for the oxidation of hypoxanthine in the presence of XO can be summarized by the following equation:

$$\text{Hypoxanthine} + 2O_2 \xrightarrow{XO} \text{Uric acid} + 2H_2O_2$$

Therefore, H_2O_2 could be electroreduced by the PB modified electrode at the applied potential of 0.05 V versus internal reference.

Fig. 2 illustrates linear calibration curve of the PB-SPE/XO/Nafion biosensor upon successive addition of different concentrations of hypoxanthine in a stirred buffer solution under the optimal conditions. Inset of Fig. 2 display a typical amperometric response of PB-SPE/XO/Nafion biosensor. The response of the biosensor at +0.05 V increased and reached a steady state. The response time (reaching 95% of the steady state) is within 8-10s, which indicates a fast process. A linear relationship between the current and hypoxanthine concentration in the range of 5 µM to 45 µM with a correlation coefficient of 0.987 and a limit of detection of 0.4 µM (signal/noise [S/N] =3) was observed. The biosensor displayed a high sensitivity of 600 mA M^{-1} cm^{-2} to hypoxanthine, which was higher than 220 mA M^{-1} cm^{-2} reported by Shan et al, 2009.

3.4. Inhibition by Allopurinol

Allopurinol is a potent inhibitor of xanthine oxidase with a reported constant of inhibition K_i 630 pM and can be clinically used for gout treatment [23].

The amperometric measurement of our constructed SPE-PB/XO/Nafion electrode was tested in the presence of 40 µM hypoxanthine (substrate) at a fixed potential of +0.05 V. A significant decrease in the current was observed after 40 µM hypoxanthine being spiked into a cell containing PBS (pH 7.5, 0.05 M + 0.1 M KCl). Upon the addition of 10 µM allopurinol (Fig. 3a) and 0.125 µM allopurinol (Fig. 3b) to the solution containing hypoxanthine, the catalytic current increased dramatically, indicating that allopurinol inhibits the activity of the immobilized XO.

The response time t_{95} (reaching 95% of the steady state) of allopurinol was prolonged to about 12 min, compared with the response time of hypoxanthine which was less than 10 s. This behavior indicates a slow process, which may be explained by the fact that allopurinol should first be oxidized by XO to its active metabolite oxypurinol that binds tightly to a partially reduced form of XO, thereby eliminating its catalytic activity. However, oxypurinol has very little affinity for the fully oxidized form of the enzyme [23], which may explain the extension in the response time.

Fig. 4 showed a typical inhibition calibration curve for the determination of allopurinol. Xanthine oxidase inhibition increased significantly with the addition of allopurinol, which decreased the production of H_2O_2. This decrease is proportional to the concentration of inhibitor. A maximum of inhibition of about 95% was obtained for 10 µM allopurinol. The linear range of allopurinol concentration is obtained up to 2.5 µM. The coefficient of variation (CV) was estimated to be 5%. I_{50} corresponding to the concentration of allopurinol that lead to 50% of degree of inhibition was estimated to be 1.8 µM from the data in Fig. 4. This value is quite near to I_{50} value of 0.2-50 µM reported in literature for free XO [4]. Indicating that the affinity of allopurinol to the proposed biosensor is very strong.

3.5. Screening of plant extracts for XO inhibitory activity

In the present study, 13 medicinal plants were selected based on their traditional use to treat gout or rheumatism by the native people of the region and were successively

extracted with hot water to give 13 extracts. All of these extracts were tested for their XO inhibition to identify potential inhibitor for gout. The assay was carried out with different dilutions of the water plant extracts. The inhibition of xanthine oxidase resulted in decreased production of uric acid was measured spectrophotometrically.

The preliminary screening of the plant extracts by spectrophotometric method revealed the presence of the inhibitory effect on the XO activity. On the extracts assayed, 11 extracts (85%) demonstrated XO inhibition, among which 7 (54%) showed a degree of inhibition greater than 50% (Table 1). The degree of inhibition of plant extracts against XO was as followed: *Mentha spicata (L.)* > *Lavandula angustifolia* > *Caryophyllus aromaticus (L.)* > *Hyssopus officinalis (L.)* > *Rosmarinus officinalis (L.)* > *Solidago Virgaurea (L.)* > *Zea mays (L.)* > *Artemisia herba-alba* > *Melissa officinalis (L.)* > *Ginkgo biloba (L.)* > *Thymus vulgaris (L.)* Moreover, at a dilution of 1/30, *Mentha spicata (L.)* showed an inhibition of over 60%. The water extract possessing XO inhibitory activity less than 50% were *Artemisia herba-alba* (49.2%), *Ginkgo biloba (L.)* (48.5%), *Melissa officinalis (L.)* (49.2%), and *Thymus vulgaris (L.)* (42.5%) (Table. 1).

The amperometric response of SPE-PB/XO/Nafion biosensor when injecting different dilutions is shown in Fig. 5a for *Mentha spicata (L.)* extracts, and in Fig. S1 (a) for *Lavandula angustifolia* and Fig. S2 (a) *Hyssopus officinalis (L.)* extracts. The degree of inhibition as a function of plant extracts dilution is illustrated in the typical inhibition calibration curve in Fig. 5b, Fig. S1 (b) and Fig. S2 (b), in which the concentration of substrate (HX) is fixed at 40 µM. The coefficient of variation was estimated to be 5%. The response time t_{95} (reaching 95% of the steady state) of the plant extracts was less than 10 s compared to the response time of allopurinol that was 12 min. It indicates a faster inhibition process of those plant extracts compared to allopurinol, which confirm that there is no limitation problems due to the diffusion of substrate through enzymatic membrane.

The evaluation of XO inhibitory activity of the selected plants using distilled water as the extraction solvent was conducted at different dilution, at which also 85% of the extracts were found to have XO inhibitory activity. This shows the fact that

Moroccan medicinal plants have well diverse chemical structure from their secondary metabolite [24], which makes them promising remedies for gout.

The degree of inhibition of XO of all extracts obtained by using distilled water as extraction solvent were listed in Table. 1. All the extracts (85%) showed a degree of inhibition greater than 50% in the case of amperometric measurements, while in the spectrophotometry measurements only 54% showed a degree of inhibition higher than 50%. The degree of inhibition of plant extract was as followed *Rosmarinus officinalis (L.)* > *Melissa officinalis (L.)* > *Solidago Virgaurea (L.)* > *Hyssopus officinalis (L.)* > *Mentha spicata (L.)* > *Lavandula angustifolia* > > *Thymus vulgaris (L.)* > *Caryophyllus aromaticus (L.)* > *Artemisia herba-alba* > *Zea mays (L.)* > *Ginkgo biloba (L.)*. In addition, at a dilution of 1/30, two plant extracts *Mentha spicata (L.)* and *Melissa officinalis (L.)*, showed an inhibition degree higher than 60% and 70% respectively.

This study shows that electrochemical method can be used as alternative of spectrophotometric method in the screening of medicinal plants for XO inhibitors. Both methods, electrochemical and spectrophotometric showed similar results for the plant possessing XO inhibitors. Moreover, the degree of inhibition obtained for the tested medicinal plants with our proposed biosensor was higher (with average of 28%) compared to spectrophotometric method, which suggest that the electrochemical method is a more sensitive method for detection of XO inhibitors compared to spectrophotometric method.

Of all the plant species under evaluation, *Mentha spicata (L.)* and *Melissa officinalis (L.)* have shown promising future to be utilized as a new natural source of XO inhibitors and an alternative to allopurinol. Moreover, these two plants are ubiquitous and can be found in almost every corner of Morocco. Although these two plants are used in Moroccan folk medicine to treat inflammatory diseases or hypertension [24-25], this is the first report on their XO inhibitory activity.

The water has shown a good capacity to extract XO inhibitors from all parts of plants. 85% of all plant extracts have demonstrated more than 50% of degree of inhibition under this solvent, this is probably due to the nature of biological active

components (alkaloids, flavonoids, terpenoids, tanins, etc) and this extraction may have produced a greater number of active constituents responsible for XO inhibition.

Still there are numerous conventional extraction techniques based on organic solvent like methanol or ethanol have been applied to the extraction of XO inhibitors from medicinal plants [1,17,26-27], these methods result to undesirable effects on the environment. Unlike methanol and ethanol, water is readily available, practically applicable, many substance dissolve in it and relatively cheap, which make it an excellent extraction solvent. In fact, some studies have applied water for the extraction of XO inhibitors [1,17]. Moreover, the infusion of medicinal plants is the traditional method employed by local population in Morocco to treat gout.

3.6. The storage stability of the biosensor

The storage stability of the developed biosensor prepared under the optimum conditions was measured for a period of 6 weeks at constant hypoxanthine concentration (40 µM). When not in use, the enzyme electrodes were kept in 0.05 M PBS (pH 7.5) at 4°C. The result of storage stability during this period is plotted in Fig. 6. The SPE-PB/XO/Nafion electrode exhibited a good stability during 3 weeks. Indeed, the amperometric response remained about 90% of its initial response. There was a rapid decrease of 40% from the initial response at the 4^{th} week. This indicates that the developed biosensor can be used for long time.

4. Conclusion

The development of an amperometric biosensor for the detection of XO inhibitors from medicinal plants was described in this paper. In our amperometric measurement, we observed that PB deposited on the SPEs electrodes had an excellent catalytic activity on the electro-reduction of H_2O_2. The developed hypoxanthine biosensor, exhibited also a good analytical characteristics such as rapid response, high sensitivity (600 $mA.M^{-1}.cm^{-2}$), selectivity, lower detection limit of 0.4 µM and a good stability during 3 weeks under the optimal conditions. Moreover, compared to traditional spectrophotometric method, the strength of the biosensor is that it is very simple, selective, low cost stable and provides rapid results. The proposed biosensor can be used in the evaluation of enzyme inhibitors from other medicinal plants and also for the screening of new active constituents that

can be used as new drugs by using only the infusion of plants. Finally, in this study the screening of medicinal plants for XO inhibitors indicates two potential plants, namely *Mentha spicata (L.)* and *Melissa officinalis (L.)* that may be useful for treatment of gout. This provides the basis for further investigation on these plants to isolate active constituents and drug development.

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Figure Captions

Fig. 1. Effect of pH on the response to 40-µM hypoxanthine in 50 mM phosphate buffer solution, 0.1 M KCl at 25°C. The operating potential is +0.05V vs Ag/AgCl.

Fig. 2. Calibration curve obtained with SPE-PB/XO/Nafion modified electrode in 50 mM phosphate buffer, 0.1 M KCl pH 7.5 at +0.05 V vs Ag/AgCl. Standard errors indicated by bars of three repeated measurements (n=3). Inset shows a typical response to successive hypoxanthine injections.

Fig. 3. Amperometric response recorded using SPE-PB/XO/Nafion in presence of 40-µM hypoxanthine (a) 10 µM allopurinol, (b) 0.125 µM allopurinol. Applied potential +0.05 V vs Ag/AgCl. Supporting electrolyte 50 mM phosphate buffer, 0.1 M KCl pH 7.5.

Fig. 4. Calibration curve of allopurinol obtained with SPE-PB/XO/Nafion modified electrode in 50 mM phosphate buffer, 0.1 M KCl pH 7.5 at +0.05 V vs Ag/AgCl. Standard errors indicated by bars of three repeated measurements (n=3).

Fig. 5. (a) Amperometric response and (b) calibration curve recorded using SPE-PB/XO/Nafion in presence of different dilution of extracts of *Mentha spicata (L.)*. Applied potential +0.05 V vs Ag/AgCl. Supporting electrolyte 50 mM phosphate buffer, 0.1 M KCl pH 7.5 containing 40-µM hypoxanthine. Standard errors indicated by bars of three repeated measurements (n=3).

Fig. 6. Storage stability of the biosensor at 0.05 V in 50 mM phosphate buffer pH 7.5 in the presence of 40-µM HX.

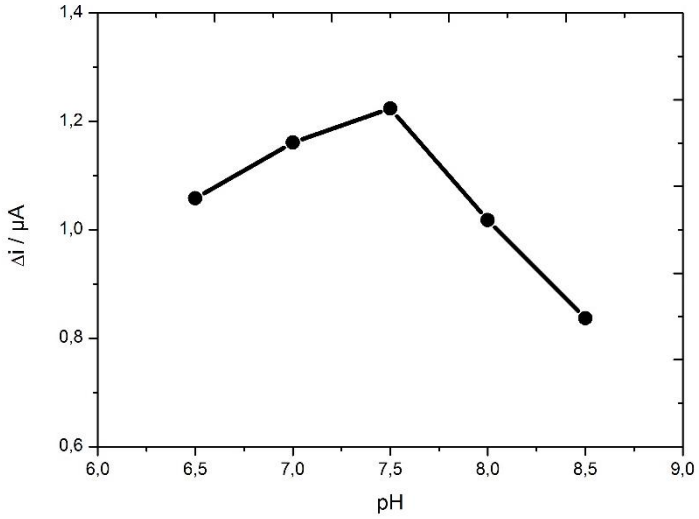

Fig. 1

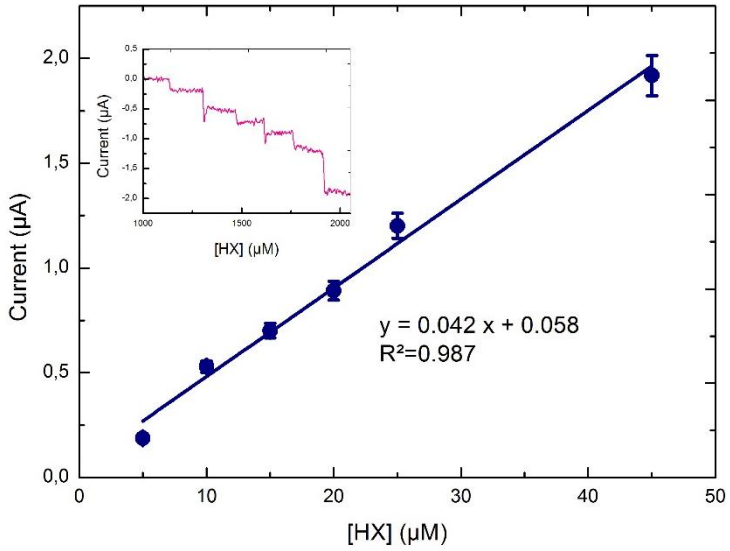

Fig. 2

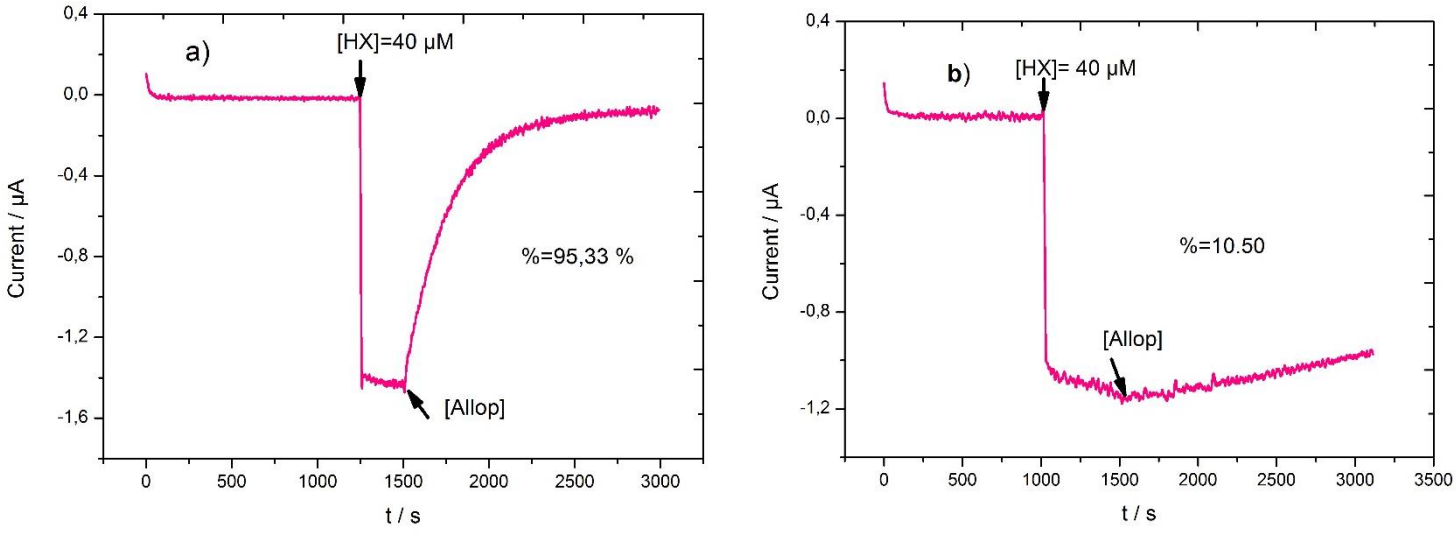

Fig. 3

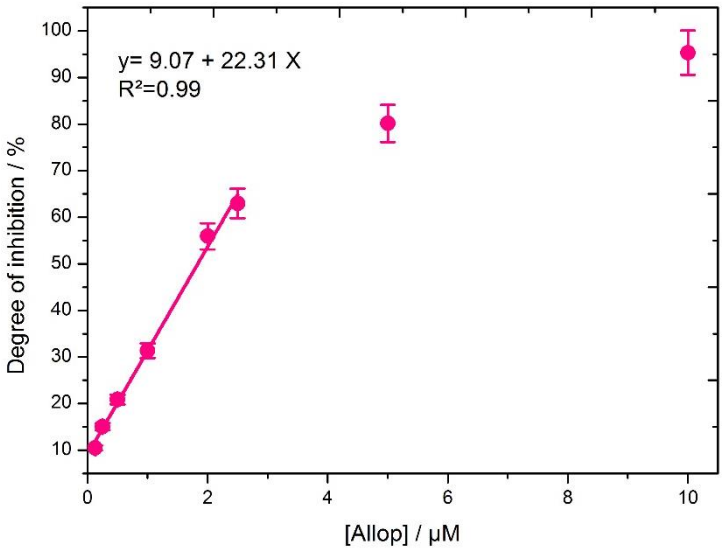

Fig. 4

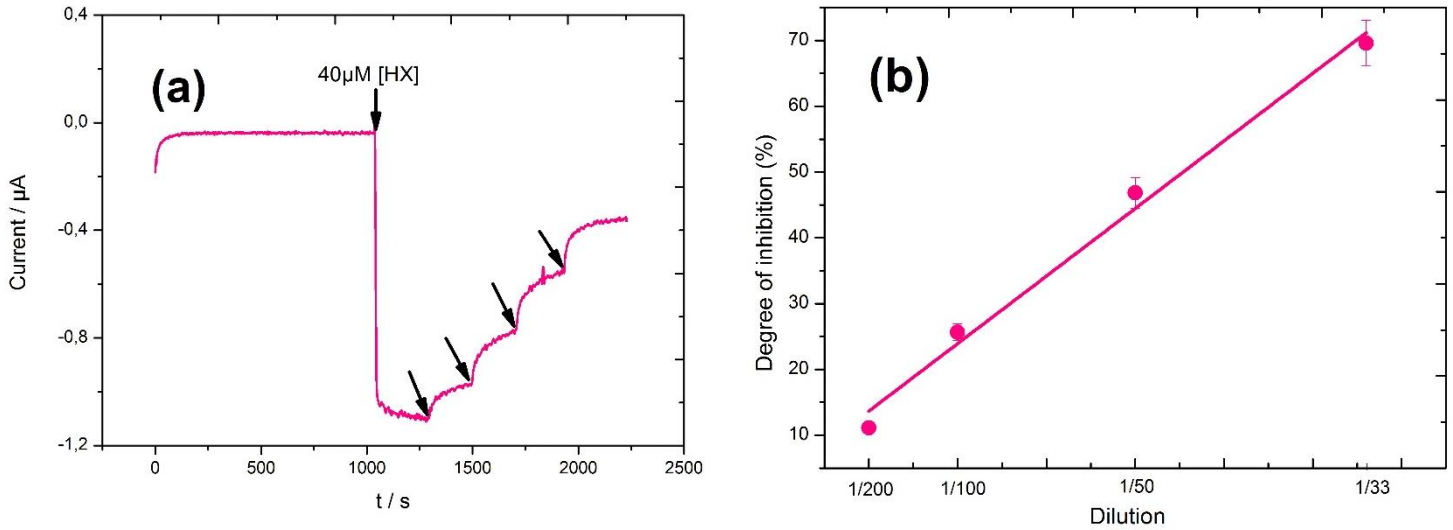

Fig. 5

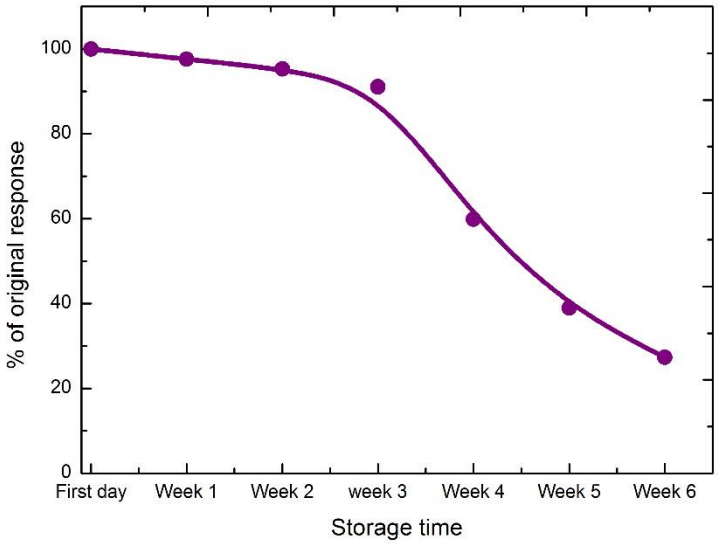

Fig. 6

Tables

Table 1. Results of the XO inhibition screening of 13 medicinal plants used traditionally in Morocco for the treatment of gout

PLANT NAME	PART USED	DILUTION	DEGREE OF INHIBITION % OF XO BY SPECTROPHOTOMETER	DEGREE OF INHIBITION % OF XO BY ELECTROCHEMISTRY
AILANTHUS ALTISSIMA	Flowers	-	-	-
ARTEMISIA HERBA-ALBA	Flowers	1/20	49.2	64.3
CARYOPHYLLUS AROMATICUS (L.)	Flowers	1/5	59	65
GINKGO BILOBA (L.)	Leaves	1/5	48.5	50.4
HYSSOPUS OFFICINALIS (L.)	Flowers	1/20	55.3	73.4
LAVANDULA ANGUSTIFOLIA	Flowers	1/20	63.3	67.2
ME LISSA OFFICINALIS (L.)	Leaves	1/30	49.2	77.7
MENTHA SPICATA (L.)	Leaves	1/30	63.5	69.6
ROSMARINUS OFFICINALIS (L.)	Leaves	1/10	52.5	77.9
SOLIDAGO VIRGAUREA (L.)	Leaves	1/15	51.9	77.4
THYMUS VULGARIS (L.)	Leaves	1/20	42.5	66.4
URTICA DIOICA (L.)	Leaves	-	-	-
ZEA MAYS (L.)	Stigma	1/10	51.4	61.9