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An optimized cupric reducing antioxidant capacity (CUPRAC) method for assessment of xanthine oxidase inhibition activity

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Abstract: This protocol clarifies a simple and precise method for measuring the activity of xanthine oxidase (XO) enzyme inhibitor. XO enzyme, which accelerates oxidative stress-related disorders through its capacity to generate hydrogen peroxide and superoxide anion radicals ($O_2^{\bullet-}$), has been found to be inhibited by several plant extracts. Enzyme samples were incubated with a suitable buffer containing adequate amounts of xanthine as a substrate to determine XO activity. The method depends on direct measurements of uric acid and hydrogen peroxide production to test XO with and without interference. The CUPRAC reagent ($Cu(Nc)_2^{2+}$) was used to inhibit enzyme reaction after incubation was complete. The generated urate and peroxide reduced the Cu(II)-neocuproine complex ($Cu(Nc)_2^{2+}$) to a brightly colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$), which was assessed with a spectrophotometer at 450 nm. XO activity was found to be directly related to the increased absorbance of the colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$). To eliminate catalase enzyme interference, the proposed method used sodium azide and was validated against XO activity using the UV method in matched samples with t-test analysis. The proposed assay can determine XO activity with high precision, as indicated by the correlation coefficient ($R^2 = 0.9935$) from comparison with the reference protocol.

Key words: CUPRAC method, neocuproine complex, xanthine oxidase, catalase, polyphenolics, xanthine oxidase inhibitor

1. Introduction

Human physiopathological conditions are often associated with reactive oxygen species (ROS) such as singlet oxygen, superoxide radical anions, hydrogen peroxide, and hydroxyl radicals.[1] A number of neurodegenerative disorders including brain and heart reperfusion damage, Alzheimer's dementia, atherosclerosis, and Parkinson's disease have been linked to oxidative stress, which is generated by an imbalance between antioxidant systems and oxidant production, including ROS[2], and the pathogenesis of inflammation, cancer, arthritis, and aging.[2,3] ROS are created in human tissues as a result of exposure to exogenous substances in the environment such as gamma rays, UV light, and x-rays, or during metal catalyzed reactions[4] and some endogenous metabolic activity involving bioenergetics electron transfer and redox

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enzymes.[5] Xanthine oxidase (XO), an enzyme that catalyzes conversion of hypoxanthine to xanthine and xanthine to uric acid, is another biological source of superoxide radicals. In both processes, superoxide anions and hydrogen peroxide are formed when molecular oxygen is reduced.[6,7] Polyphenols such as flavonoids have been shown to have antiallergenic, antiviral, anti-inflammatory, and vasodilating effects in several studies. Flavonoid antioxidant activity is associated with these pharmacological properties. Flavonoid protective properties are attributed to the ability to inhibit ROS generation by blocking specific enzymes or chelating transition metals implicated in free radical formation, scavenging radical species, particularly ROS, and increasing antioxidant defense regulation.[8,9]

XO is a complex molybdoflavoprotein that is primarily present in dehydrogenase form; only the oxidase form participates in production of considerable superoxide $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2). XO is a key biological $O_2^{\bullet-}$ producer associated with an ischemia and different forms of vascular damage, chronic heart failure, and inflammatory diseases.[10] XO is responsible for uric acid production; its presence at elevated concentrations may indicate increased oxidative stress and consequent risk. Gout is caused by high concentrations of uric acid in the blood, known as hyperuricemia.[11] The kidneys and gastrointestinal tract are responsible for final elimination.[12] Increases in serum uric acid, oxidative stress, endothelial dysfunction, and left ventricular dysfunction, all of which have been associated with the etiology of heart failure,[13] may result from increased XO pathway activity.[14,15]

Inhibition of XO reduces tissue and vascular damage resulting from oxidative stress. Furthermore, it lowers uric acid concentration, a risk factor for development of all types of cardiovascular disease.[16] Xanthine oxidase inhibitors (XOIs) have demonstrated effectiveness in treatment of hepatitis, brain tumors, and gout.[17,18] XOIs may be effective in the treatment of a variety of other diseases.[19,20]

Researchers have provided many protocols to measure XO enzyme activity. The most common protocol is based on measuring the change in absorbance in the ultraviolet region by following the increase in absorbance at 295 nm caused by the formation of urate[21,22] or by the production of NAD(P)H.[23] Another protocol is based on measurement of the disappearance rate of xanthine at 270 nm.[24] Atlante *et al*.[25] described a fluorometric protocol to measure XO activity. As a consequence of pterine oxidation by the produced hydrogen peroxide, the process was used to synthesize fluorescent isoxanthopterin from non-fluorescent pterine (Ex/Em: 345 nm/390 nm).

A novel protocol for assessing XO activity was developed using the oxidation of 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonate) (ABTS) by peroxidase and uricase.[26] After 10 min at 410 nm, the increase in absorbance of the oxidized form of ABTS was proportional to XO activity. Naoghare *et al*.[27] developed a high-throughput chip-based protocol using a photodiode array (PDA) microchip technique to examine the inhibitory effects of pharmacological analogs on XO. The test used the red light absorption properties of nitroblue tetrazolium (NBT) formazan, which is produced when NBT is reduced by free radicals.

Liu *et al*.[28] devised an ultrahigh-performance liquid chromatography and triple quadrupole mass spectrometry (UHPLC-TQ-MS) technique with improved accuracy and speed by adding WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt) to the XO enzymatic reaction. Using digital automation, the methodology was applied to test the XO inhibitory properties of a range of herbal extracts and components from natural sources.

Özyürek *et al.*[23] used the cupric reducing antioxidant capacity (CUPRAC) spectrophotometric protocol to measure the XO-inhibitory activity of polyphenols (XOI-CUPRAC method). The method used direct measurements of hydrogen peroxide and uric acid production to assess XO with and without interference. The CUPRAC absorbance of the enzymatic reaction solution decreased in the presence of polyphenols owing to the reduction of Cu(II)-neocuproine reagent (Cu(II)-Nc) by the products of the xanthine-xanthine

oxidase system, with the difference proportional to the XO inhibition ability of the investigated compound.

The proposed modified protocol considers two types of interference related to the previous CUPRAC protocol. The first is due to the presence of catalase enzyme, which breaks down hydrogen peroxide resulting from XO enzyme activity. The second interference is the result of natural products such as polyphenols used to examine XO enzyme inhibition. The proposed assay uses a simple protocol to exclude the interferences in the XOI-CUPRAC method. The protocol is simple, fast, and reliable for testing XOI activity. The test can be used as an evaluation method, and is appropriate for research purposes. The validation procedure demonstrated that the diagnostic method is suitable for many types of biological samples.

2. Materials and Methods

2.1. Chemicals

Ammonium acetate, copper(II) chloride, dipotassium phosphate (K_2HPO_4), hydrochloric acid, hydrogen peroxide (30 %), perchloric acid, trichloroactetic acid, sodium hydroxide, and sodium azide were purchased from Merck (Burlington, Massachusetts, United States). 2,2'-Azino-di(3-ethylbenzthiazoline-6-sulphonate) (ABTS), neocuproine (Nc) (2,9-dimethyl-1,10-phenanthroline), peroxidase (horseradish), uricase, xanthine oxidase (grade 1, ammonium sulfate suspension), and xanthine were obtained from Sigma-Aldrich (Missouri, United States). The purity of all chemicals that used in the protocol exceeds 99 %.

2.2. Instrument

A UV-visible spectrophotometer (PG T80$^+$, England) was used to measure the spectra in the protocol.

2.3. Reagents

Phosphate buffer solution (pH 7.8, 100 mM) was prepared by dissolving 13.61 g of monopotassium phosphate (KH_2PO_4 MW, 136.09 g/mol) and 3.62 g of sodium hydroxide (NaOH MW, 40.00 g/mol) in 800 mL of distilled water. The pH of the solution was adjusted using HCl or NaOH. The final volume was increased to 1 L with distilled water. Xanthine solution (10 mM) was prepared by dissolving 0.1521 g of xanthine in 70 mL of sodium hydroxide (25 mM) and 30 mL of KH_2PO_4 (66.7 mM). Sodium azide (25 mg) was added. The final pH was adjusted to 7.8. Phosphate buffer solution was used to prepare standard hydrogen peroxide (2 mM) (pH 7.8, 100 mM). At 240 nm, a molar extinction value of 43.6 M^{-1} cm^{-1} was used to adjust the final concentration. Copper(II) chloride (100 mM) was prepared with 0.4262 g of $CuCl_2 \cdot 2H_2O$ dissolved in 250 mL of distilled water. Ammonium acetate buffer (NH_4Ac) (pH 7.0, 1.816 M) was prepared with 35 g of NH_4Ac dissolved in 250 mL of distilled water. Neocuproine (2,9-dimethyl-1,10-phenanthroline) (Nc) (7.5 mM) was prepared with 0.039 g of Nc dissolved in 25 mL of 96 % ethanol. Fresh working reagent (CUPRAC reagent) was prepared by mixing volumes of the prepared reagents Cu(II):Nc:NH_4Ac at a ratio of 1:1:2 (v/v/v). XO solution was prepared by diluting 3 U/mg solid of original XO suspension with PBS (pH 7.8, 100 mM) to a final concentration of 0.04 U/mL. The perchloric acid solution 3.2 % (w/v) was prepared with 3.2 mL of perchloric acid dissolved in a suitable volume of distilled water in a 100-mL volumetric flask. ABST reagent solution was prepared by mixing 2 mmol/l ABTS and 2500 U/l peroxidase in 1L of pH 7.8 phosphate buffer. Undiluted uricase (10 U/mg) was utilized.

Plant extracts were utilized to examine XOI activity. Five plant extracts rich with polyphenols were prepared using a home blender. The group of plants consists of *Trigonella spp., Portulaca grandiflora, Myrtus communis, Passiflora caerulea, and Hibiscus sabdariffa*. Fresh leaves (50 g) were mixed with buffer phosphate solution (950 mL, pH 7.8), and the final volume was adjusted to 1 L. The total polyphenol concentration was estimated using a spectrophotometric method.[29] Each extract was prepared in a range of concentrations based on its polyphenol content.

2.4. Procedure

2.4.1. Standard method for assessment of XOI

The XO activity was measured spectrophotome-

trically at 293 nm by detecting the production of uric acid. Potassium phosphate buffer (100 mM, pH 7.8), 75 mM xanthine, and 0.04 units of XO were used in the enzymatic reaction. The decrease in uric acid production at 293 nm was used to test the inhibition of XO activity with different inhibitors. The enzyme was preincubated for 5 min with the test chemical, which was dissolved in an appropriate buffer, before the reaction was started using xanthine. The herbal extract inhibition ratio was calculated using Eq. (1).

$$\text{Inhibition ratio (\%)} = 100*[(A_0 - A)/ A_0] \quad (1)$$

where A_0 and A are the absorbances of the system in the absence and presence of the inhibitor, respectively.

2.4.2. ABTS Procedure

In a centrifuge tube, a 1.0 mL substrate-buffer solution, 5 µl of uricase, and 25 µl of enzyme sample, 25 µl of antioxidant sample solution, are mixed and incubated for 10 minutes at 30°C. One mL of ABST reagent solution followed by one mL of 2 M $HClO_4$ were added and vortexed. The solution was centrifuged for 5 minutes at 3000 RPM. The absorbance of the supernatant is measured at 410 nm. The herbal extract inhibition ratio was calculated using Eq. (1).

2.4.3. CUPRAC method for assessment of XOI

A mixture of 0.5 mL of 0.5 mM xanthine, 0.2 mL of antioxidant sample solution, 1.8 mL of 1:9 EtOH–PBS (pH 7.8) (v/v), and 0.2 mL of 0.04 U/mg XO was added to a test tube in this order. The mixture was incubated in a water bath at 37 °C for 30 min in a total volume of 2.7 mL. After 30 min, the reaction was terminated by vortexing for one minute with 0.1 mL of 3.2 % perchloric acid solution.

Fresh CUPRAC reagent was added to 0.2 mL of the incubation solution in the following order: (V total = 1.0 mL) 0.2 mL Cu(II) + 0.2 mL Nc + 0.4 mL NH_4Ac buffer + 0.2 mL incubation solution. The absorbance was measured after 30 min against a reagent blank, with and without an inhibitor. The average of three experiments was used for calculation.

The inhibition ratio (%) of the herbal extract was calculated using Eq. (2).

$$\text{Inhibition ratio (\%)} = 100*[(A_0 - A)/ A_0] \quad (2)$$

where A_0 and A are the CUPRAC absorbances of the enzymatic solution in the absence and presence of the inhibitor, respectively.

2.4.4. Modified CUPRAC method for assessment of XOI

The modified XOI–CUPRAC method is presented in *Table* 1.

The inhibition ratio of the herbal extract (%) was calculated using Eq. (3):

$$A_{\text{corrected}} = At_{30} - At_0 \quad (3)$$

$$\text{Inhibition ratio (\%)} = 100*[(A_0 - A_{\text{corrected}})/ A_0] \quad (4)$$

where A_0 is the absorbance of the control tube; At_0 and At_{30} are the absorbances of the test tube at t = 0 and t = 30 min, respectively.

Table 1. Details of modified XOI–CUPRAC method

Reagent	Test (t_{30})	Test (t_0)	Control	Blank
Xanthine	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Antioxidant sample	0.2 mL	0.2 mL	-------	-------
1:9 EtOH–PBS mixture (pH 7.8) (v/v)	1.8 mL	1.8 mL	2.0 mL	2.2 mLmL
Perchloric acid solution (3.2 %)	------	0.1 mL	------	------
XO solution	0.2 mL	0.2 mL	0.2 mL	------
The test tubes were incubated at 37 °C for 30 min.				
Perchloric acid solution (3.2 %)	0.1 mL	------	0.1 mL	0.1 mL

Following incubation, fresh CUPRAC reagent was added to 0.2 mL of incubation XOI solution as follows: (V total = 1.0 mL) 0.2 mL Cu(II) + 0.2 mL Nc + 0.4 mL NH_4Ac buffer + 0.2 mL incubation XOI solution. The absorbance was measured at 450 nm after 30 min against a reagent blank. The average of three experiments was used for calculation.

2.5. Validation the modified method

The proposed method was evaluated using the UV method, as previously reported by Dew *et al*..[21]Three duplicates of five plant extracts were examined to determine intra- and inter-day precision and accuracy. The preparation of plant extracts were prepared as described in material and method section. A suitable series of concentrations of quercetin and catechin were used as standards. The data were analyzed using GraphPad Prism v.8 software (San Diego, CA, USA).

3. Results and Discussion

3.1. Modified CUPRAC method for assessment of XOI

This study presents a simple protocol for evaluating XOI activity using the CUPRAC method. The protocol depends on the reduction of ($Cu(Nc)_2^{2+}$) to a brightly colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$) by XO products (uric acid and hydrogen peroxide); the resulting solution was measured by spectrophotometry at 450 nm. XO activity is related to the incremental absorbance. In *Fig.* 1, the proposed assay is based on XO products used to measure XO activity. The resulting CUPRAC complex ($Cu(Nc)_2^{+}$) produces a single peak at 450 nm. The absorbance was proportional to the concentration of uric acid and hydrogen peroxide formed as a result of XO activity (*Fig.* 1). The number of micromoles of uric acid or hydrogen peroxide generated per unit time represents one unit of XO enzyme.

The method was originally described by Özyürek *et al*.[23] to assess XOIs. The modified protocol considers two types of interference; the first is caused by the presence of the catalase enzyme, which breaks down hydrogen peroxide resulting from XO activity. After studying the key role of catalase in the resulting CUPRAC color, Özyürek *et al*.[23] concluded that the presence of catalase disrupted the absorbance signal as a result of Cu(I)–Nc. With high catalase activity, the CUPRAC peak produced by the presence of hydrogen peroxide may be totally suppressed. Özyürek *et al*.[23] reported that the CUPRAC method may be used to evaluate a possible XOI by assessing the CUPRAC absorbance of the xanthine-xanthine oxidase (X–XO) conversion products without hydrogen peroxide degradation. Despite the conclusions reached in the study, the interference of catalase present in fresh plant samples remained, and was used to assess XO inhibition activity. The current method avoided catalase interference by using sodium azide as a selective inhibitor, as shown in *Scheme* 1. Sodium azide was used effectively to prevent catalase interference in XO assessment.[30] Hydrogen peroxide is

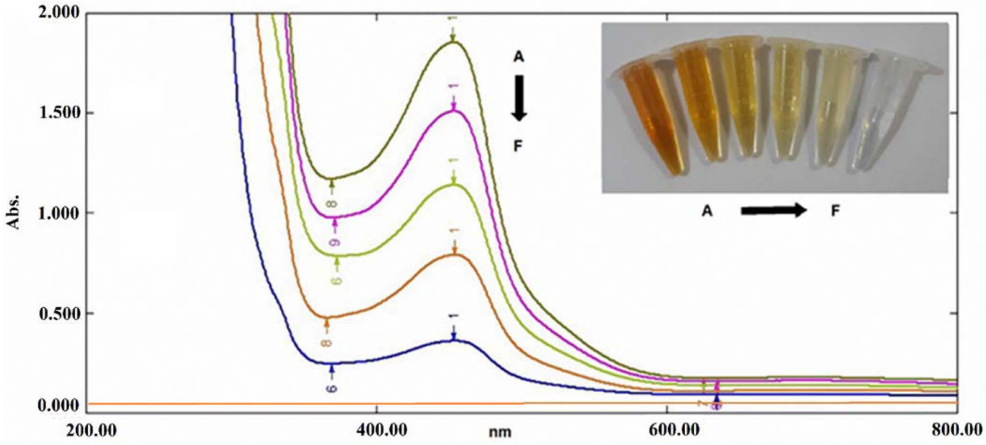
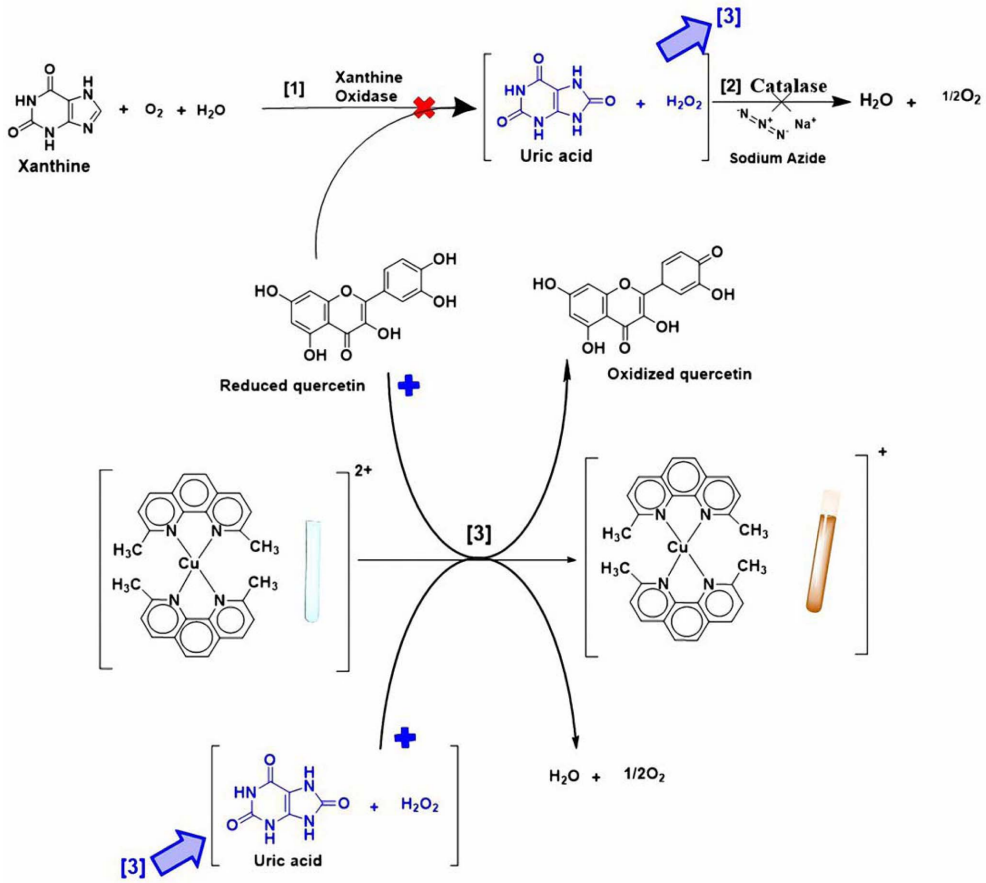


Fig. 1. Spectrophotometric characteristics of the resulting Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$) were shown to be associated with XO enzyme activity. Absorption spectra were obtained by reducing ($Cu(Nc)_2^{2+}$) to a brightly colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$) as a result of the formation of hydrogen peroxide and uric acid from the XO enzyme reaction. The resulting complex was measured by spectrophotometer at 450 nm: (A to F) represent (10, 8, 6, 4, 2, 0) U of XO enzyme activity.



Scheme 1. Cu(I)-neocuproine chelate is formed by the interaction of Cu(II)-neocuproine complex with uric acid and hydrogen peroxide (λmax = 450 nm). Uric acid and hydrogen peroxide formed as a result to xanthine oxidase activity (reaction 1). They were utilized to reduce ($Cu(Nc)_2^{2+}$) to a brightly yellow colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$) (reaction 3). To prevent the consumption of hydrogen peroxide, sodium azide was used to inhibit catalase enzyme (reaction 2). Polyphenol such as quercetin was applied to inhibit xanthine oxidase activity. At the same time, polyphenol may reduce ($Cu(Nc)_2^{2+}$) to a brightly yellow colored Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$) (reaction 3). ✚ Refers to compounds that cause an increase in absorbance. ✖ Refers to compounds that cause decrement of absorbance.

a particular substrate for the catalase enzyme. The sodium azide, which specifically inhibits the catalase enzyme, is used in the previous enzymatic procedures to prevent interfering with the catalase enzyme.[30,31] The second interference is attributed to natural products such as polyphenols used to examine inhibition of the XO enzyme. Antioxidants such as polyphenols react with the CUPRAC reagent to produce the same color that results from reaction with the products of XO enzyme activity (uric acid and hydrogen peroxide).

Antioxidant interference that occurs with the XOI–CUPRAC method is easily prevented. Two test tubes are used: one with an enzymatic reaction time equal to 0 min (t_0), and the other with an enzymatic reaction time equal to 30 min (t_{30}). The use of a correction test tube (t_0) is necessary to eliminate interference caused by the presence of antioxidants in the sample. In the protocol, the test tube absorbance (t_{30}) is related to two types of compounds: products of XO enzymatic activity, hydrogen peroxide and uric acid, and antioxidants used to inhibit XO activity. The absorbance of the correction test tube (t_0) in the protocol was attributable only to antioxidants used to examine XO inhibition activity. We prevented interference of any

substance that would change the CUPRAC reagent absorbance by subtracting the absorbance of the correction test tube (t_0) from the absorbance of the other test tube (t_{30}). This suggests that the residual absorbance is due to products of XO enzymatic activity, hydrogen peroxide and uric acid.

3.2. Validation and reproducibility

The modified CUPRAC procedure was validated by comparing the XO-inhibitory activity of herbal extracts as IC50 values in the X–XO reaction solution with matched samples using both suggested and reference methods. All plant extracts exhibited a positive result for the catalase enzyme; activity was measured using the aniline–hydroquinone method.[30] The ability of fresh plant extracts to inhibit XO enzyme was tested. *Table* 2 indicates that the results of the proposed protocol were strongly associated with the results of the UV protocol.[21] The t-test analysis results confirmed that the current method is similar to the reference method. With the same quantities of plant extracts, the XO activity determined using the CUPRAC protocol was almost equal to that reported using the UV protocol.

3.3. Methods comparison

The XOI-CUPRAC method and the UV method were compared using the Bland-Altman analysis.[32] For mathematical calculation, Bland–Altman analysis and Passing–Bablok plot were applied by utilizing

Table 2. The XOI activity using the CUPRAC protocol (as IC50 values in g·mL^{-1}) was compared with the values obtained using the UV method on the same samples

Type of extract	XOI activity (IC50 μg mL^{-1}) for herbal samples	
	Modified CUPRAC method	UV method
	Mean ± SD (RSD%)	Mean ± SD (RSD%)
Trigonella spp.	12.07 ± 0.57	11.25 ± 1.25
Portulaca grandiflora	13.71 ± 1.25	11.55 ± 1.4
Myrtus communis	14.35 ± 0.95	15.55 ± 1.35
Passiflora caerulea	14.75 ± 0.75	15.8 ± 1.25
Hibiscus sabdariffa	11.25 ± 0.65	11.77 ± 1.35
Catechin *	2.07 ± 0.11	2.15 ± 0.09
Quercetin *	2.33 ± 0.17	2.28 ± 0.15

*XOI was measured as IC50 values in μM.

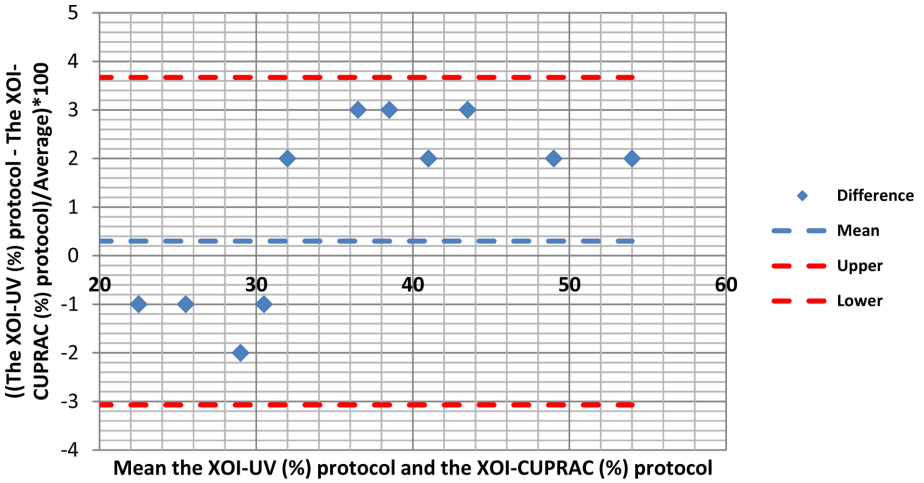


Fig. 2. The Bland–Altman plot represents the relative differences in the xanthine oxidase inhibition (%) that assessed with the XOI–CUPRAC method and UV protocol, as well as the mean relative bias.

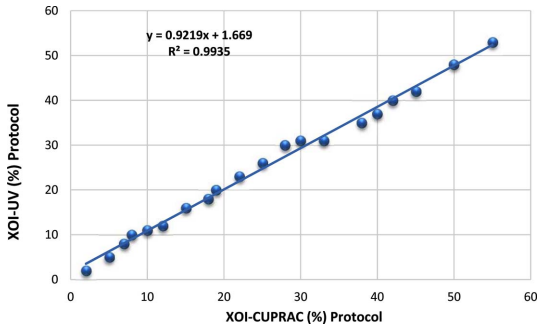


Fig. 3. XOI activity was assessed using the XOI-CUPRAC and UV protocol over a series of fresh extract dilut roselle leaves (*Hibiscus sabdariffa L.*) as an XOI.

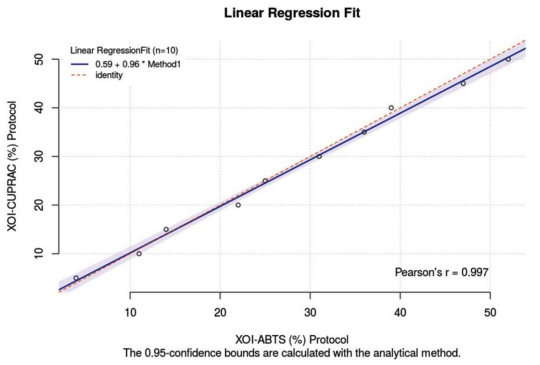


Fig. 4. Linearity of the modified XOI-CUPRAC (%) protocol for a series of dilutions of fresh extract of roselle leaves (*Hibiscus sabdariffa L.)* obtained by plotting a straight line between the modified XOI-CUPRAC (%) protocol and the XOI-ABTS (%) protocol.

the QiMacros application (Know Ware International, Denver, USA) with Microsoft Excel 2016. The Bland-Altman analysis was performed using series dilutions of fresh extract of roselle leaves (*Hibiscus sabdariffa L.*). The polyphenol concentration ranged between 0.5 and 20 μg gallic acid equivalent per mL. The Bland–Altman plot shows comparative differences between the XOI–CUPRAC and UV methods, and indicates the mean relative bias (*Fig.* 2). The correlation coefficient between the two protocols was 0.9935. Thus, the XOI–CUPRAC method is nearly as accurate as the reference procedure. Passing–Bablok similarity analysis revealed a strong association between the XOI-CUPRAC method and the UV protocol (*Fig.* 3). *Fig.* 2. The Bland–Altman plot represents the relative differences in the xanthine oxidase inhibition (%) that assessed with the XOI–CUPRAC method and UV protocol, as well as the mean relative bias.

Using a web-based software for bias assessment and analytical method comparison,[33] the examined method's linearity was assessed by comparison with the ABTS method,[26] as shown in *Fig.* 4. Limits of quantification (LOQ) and detection (LOD) were used to determine the sensitivity of the modified protocol.

From the results shown in *Fig.* 4, the linearity of the XOI–CUPRAC method was compatible with that of the ABTS protocol. The LOQ 1 % XOI and LOD 3 % XOI results confirmed the high sensitivity of the modified XOI–CUPRAC method. The linearity of the XOI–CUPRAC method was comparable with that of the ABTS and UV protocols. The XOI–CUPRAC method has many advantages over the UV protocol. The present method has three significant advantages. First, a microplate or glass cuvette may be used to measure the final absorbance. Second, the method is free of interference caused by the presence of UV-absorbing biomolecules such as amino acids, protein, and nucleic acids. Finally, to assess the concentration of hydrogen peroxide, the protocol does not require the use of expensive reagents or complicated instruments.

4. Conclusions

This study highlighted a simple method for evaluating XOI activity using only a few steps. This procedure may be used to evaluate XOI activity in different types of biological samples with high concentrations of interfering chemicals. The enzymatic reaction of XO uses the CUPRAC reagent as a probe to measure XOI activity. XO activity is associated with increasing absorbance.

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