Improved Bioautographic Xanthine Oxidase Assay: Combining HPTLC separation and activity assessment for phytopharmaceutical research

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Introduction

Xanthine oxidase (XO), a molybdenum containing flavoprotein, catalyses the oxidation of hypoxanthine and xanthine to uric acid under the formation of superoxide radicals and hydrogen peroxide. An overproduction of these reaction products in the human body is associated with diseases such as hyperuricemia, gout, hypertension, diabetes and different inflammatory diseases. Bioautography offers a rapid and simple tool for screening of secondary metabolite profiles of medicinal plants by HPTLC combined with screening of potential health beneficial activities [1]. XO inhibitory effects can be directly visualised as white zones on a purple coloured thin layer chromatogram based on the reaction of superoxide radicals with nitroblue tetrazolium chloride (NBT). The aim of this work has been to optimize and validate a bioautographic XO Inhibition assay first described by Ramallo et al. to obtain reliable and reproducible results [2]. The validated XO Assay has been tested using extracts of *Camellia sinensis* as known XO inhibitor as well as different *ex situ* and *in vitro* extracts of *Artemisia alba* [3].

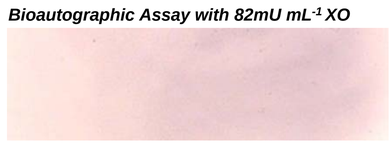
Results

Validation aspects of the Bioautographic assay

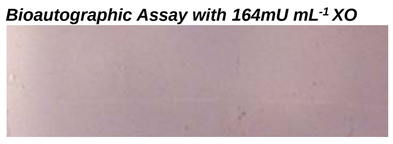
XO-Activity

Bioautographic XO-Assay with different amounts of microbial xanthine oxidase. Incubation time and temperature were adjusted according to the XO properties

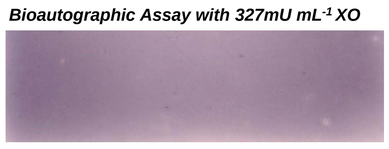
Bioautographic Assay with 82mU mL^{-1} XO



Bioautographic Assay with 164mU mL^{-1} XO



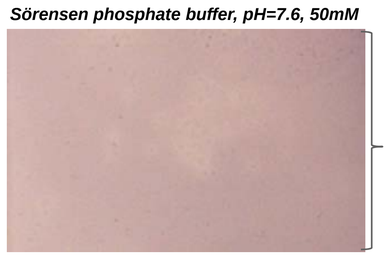
Bioautographic Assay with 327mU mL^{-1} XO



Buffer systems

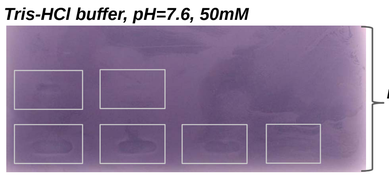
HPTLC Layer spotted with decreasing amounts of Allopurinol and stained with the XO Assay

Sörensen phosphate buffer, pH=7.6, 50mM



XO-inhibition due to Calcium ions (Ca^{2+})

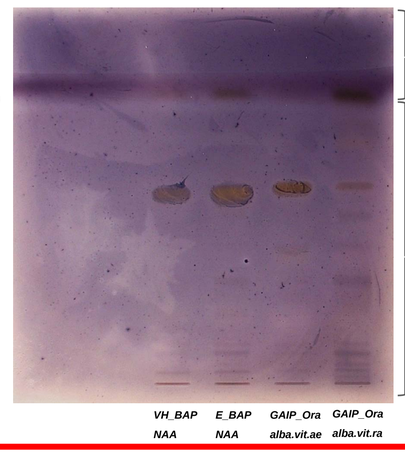
Tris-HCl buffer, pH=7.6, 50mM



False negative results

Inhibitory effects of solvent mixtures

Bioautographic assay subsequently after plate development of 10mg/mL plant extracts with Ethylacetate: Water: Acetic acid: Formic acid (100:26:11:11) (v:v) for a distance of 80 mm

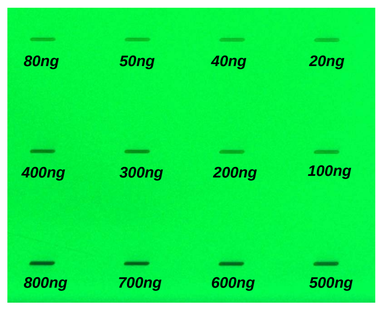


No XO-inhibitory effects

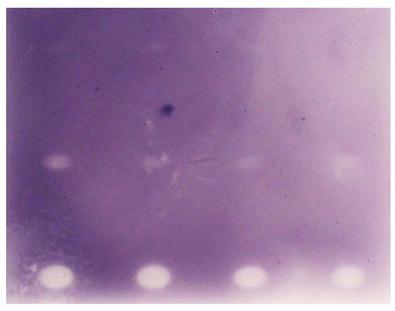
XO-inhibitory effects until 80mm

HPTLC Layer spotted with decreasing amounts of Allopurinol and stained with the XO Assay

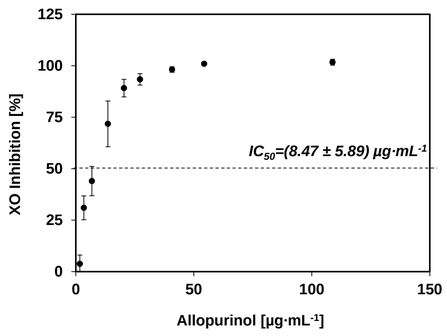
REM254



XO-Assay



Microtiter plate assay

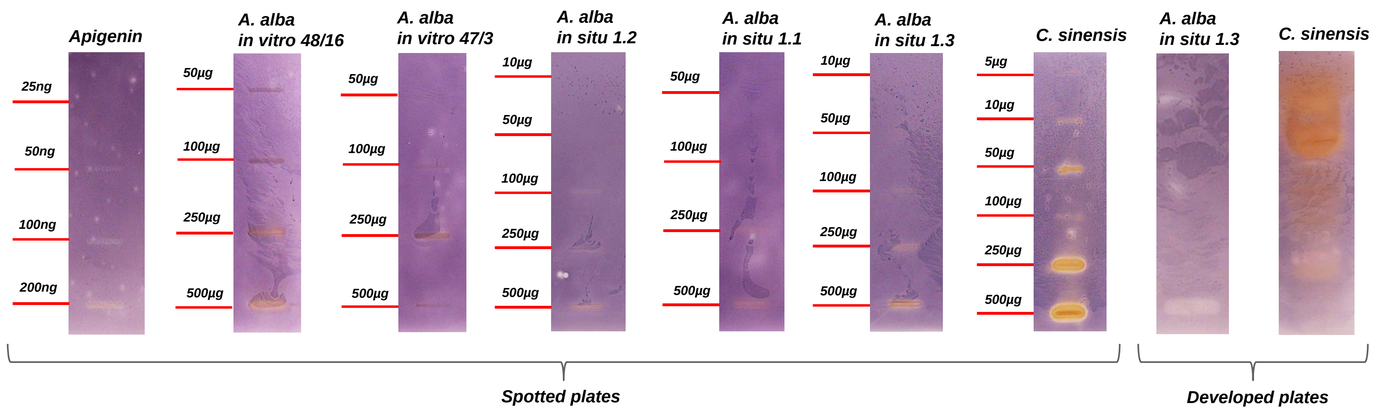


XO inhibitory properties of Allopurinol. Spectrophotometric determination of XO inhibitory activity in a microtiter plate [4].

XO Assay sensitivity

- IC_{50} was determined in a microtiter plate assay at an Allopurinol concentration of (8.47±8.89) µg·mL^{-1}
- The total amount of 271ng Allopurinol per MT well requiredfor reaching 50% XO inhibition can also be detected by using the bioautographic XO assay
- XO assay is therefore suitable for a rapid screening of constituents with strong XO inhibitory properties allowing a simple yes or no answer directly on the HPTLC plate

Bioautographic XO inhibition detection on spotted and developed HPTLC layers spotted with decreasing amounts of extracts and reference substances



Assay improvements

- Reproducible bioautographic assay procedure
- XO Assay was validated by including positive and negative control
- XO Assay scale up from 5x5cm TLC layer upon 10x10cm HPTLC plates
- Amount of XO agar solution could be reduced
- Reduction of initial xanthine oxidase activity

Conclusion and Outlook

- The optimized bioautographic Xanthine Oxidase inhibition assay is a rapid and valid research tool for the assessment of active secondary metabolites from medicinal plants.
- Allopurinol spots could be detected down to an applied amount of 50ng. Extracts of *C. sinensis* and *A. alba* showed also to contain constituents with XO inhibitory activity, that could be visually detected down to an applied amount of 10µg dry weight (dw) for *C. sinensis* extract and 100µg dw for *A. alba.* Inhibition of XO due to Apigenin could be revealed down to an applied mass of 50ng.
- Buffer systems with secondary ions such as Ca^{2+} showed to contain XO inhibitory effects. Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) interacted with the positive control Allopurinol. Most appropriate for the bioautograpphic XO assay was the use of potassium phosphate buffer at a pH of 7.6.
- Solvent residues on HPTLC plate led to a reduced plate staining when XO bioautographic assay was performed subsequently after plate development. Therefore developed HPTLC plates should be fully dried for complete removal of solvents.
- Since this assay uses superoxide radicals for the measurement of xanthine oxidase activity, superoxide radical scavengers can also generate positive results on the HTPLC plate. However, such compounds can be differentiated from pure XO inhibitors by the direct measurement of uric acid by using a standard XO microtiterplate assay [4].

Materials and Methods

Plant material

Green tea was purchased from a pharmacy. *A. alba in situ* samples (1.1; 1.2; 1.3) were collected in different regions of bulgaria. The samples were dried at 70°C and transferred to ZHAW for analysis. A. alba in vitro samples were grown on regular MS media.

Sample preparation

Dried leaves of *C. sinensis* and dried plant material of *A. alba* were extracted using Methanol and incubated for 10 min in ultrasonic bath prior to 12h extraction time in the fridge under light exclusion conditions. Before filtration all samples were temperated and shortly agitated using Vortex. All samples were stored at 4°C in the dark prior to analysis. Positive control samples were prepared freshly by dissolving Allopurinol in Methanol. Negative control sample contained Methanol only.

HPTLC equipment and conditions

Equipment: CAMAG ATS4, ADC2, TLC Visualizer and VisionCats Software. Stationary phase: HPTLC Silica Gel 60 F_{254} aluminium sheets and glas plates 10x10 cm (Merck). Separation was achieved using solvent mixture 100:26:11:11 (v:v) Ethylacetate: Water: Acetic acid: Formic Acid for a distance of 80 mm. Extract application volume was 10µl. Allopurinol at different concentrations was spotted on the HPTLC plate in an application volume of 10µl in order to identify the detection limit. All reference substabces were prepared in Methanol. Reference application volume was 10µl.

XO Microtiter plate Assay

XO Microtiter plate Assay was performed according to Havlika et al. by measuring uric acid production [4]. Absorption at 295nm was measured by a Biotek Synergy Micro Plate Reader.

Bioautographic Assay Procedure

- Low gelling temperature Agarose was dissolved at 70°C in water for injection.
- The solution was cooled to 55°C and potassium phosphate buffer (150mM, pH=7.6) was added and mixed by a magnetic stirrer
- The buffered agarose solution was given in a plastic tube containing 1.5mM nitroblue tetrazolium chloride solution
- Microbial xanthine oxidase (3.14UmL^{-1}) was added and carefully mixed by inversion
- Approximately 12mL of the solution was distributed over a 10x10cm HPTLC layer. The HPTLC plate was solidified at room temperatures in the dark for 20 min
- The plate was then immersed in 3mM xanthine solution at 45°C for 60min in the dark
- The final concentration of the assay components were 7.5mgmL^{-1} low gelling agarose, 50mM potassium phosphate buffer, 0.5mM NBT solution and 327mUmL^{-1} xanthine oxidase

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References

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