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Pitfalls in cell culture work with xanthohumol

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Xanthohumol, the most abundant prenylated chalcone in hop (*Humulus lupulus* L.) cones, is well known to exert several promising pharmacological activities *in vitro* and *in vivo*. Among these, the chemopreventive, anti-inflammatory and anti-cancer effects are probably the most interesting. As xanthohumol is hardly soluble in water and able to undergo conversion to isoxanthohumol we determined several handling characteristics for cell culture work with this compound. Recovery experiments revealed that working with xanthohumol under cell culture conditions requires a minimal amount of 10% FCS to increase its solubility to reasonable concentrations (~50–75 μmol/l) for pharmacological *in vitro* tests. Additionally, more than 50% of xanthohumol can be absorbed to various plastic materials routinely used in the cell culture using FCS concentrations below 10%. In contrast, experiments using fluorescence microscopy in living cells revealed that detection of cellular intake of xanthohumol is hampered by concentrations above 1% FCS.

1. Introduction

Triggered by the work of Gerhäuser et al. (2002) the pharmacological characterization of the prenylated chalcone xanthohumol (XAN, Fig. 1) strongly advanced in the last decade. It has been found that XAN, which is the most abundant chalcone in hop (*Humulus lupulus* L.) cones, showes among others chemopreventive (Gerhäuser et al. 2002), anti-inflammatory (Dorn et al. 2010), cytotoxic (Delmulle et al. 2006; Vogel et al. 2008) and anti-angiogenic effects (Albini et al. 2006). Mechanistically, it has been observed that XAN is able to influence the transcription factors NF-κB, NF-AT, AKT and NrF2 (Albini et al. 2006; Choi et al. 2009; Lee et al. 2011) and to trigger apoptosis (Deeb et al. 2010; Strathmann et al. 2010). Furthermore, the modulation of enzymes involved in carcinogen metabolism and detoxification has been reported (Gerhäuser et al. 2002; Dietz et al. 2005).

Interestingly, it has been noticed that handling of xanthohumol is difficult as the water solubility is low with only 1.3 mg/l at 23 °C (Stevens et al. 1999), what is always a drawback for pharmacological *in vitro* and *in vivo* studies. Moreover, the stability of XAN depends on the milieu, leading to the formation of the better water soluble flavanone isoxanthohumol under the influence of heat and water. Both aspects result in low XAN contents in beer and consequently, isoxanthohumol is much more abundant after brewing in comparison to XAN itself. As the same process is observable for the chalcone desmethylxanthohumol, which is converted to the phytoestrogen 8-prenylnaringenin (Stevens et al. 1999), stability of chalcones and their conversion to the corresponding flavanones is a topic of general importance.

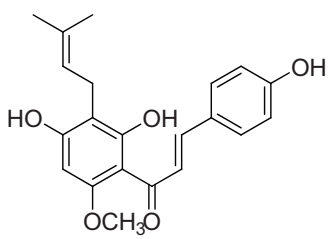


Fig. 1: Structure of xanthohumol

A very recent study showed that not only brewing processes, but also *in vitro* assay conditions can initiate instability of XAN and thus artificially influence the pharmacological activity. The presence of fetal calf serum (FCS) is supposed to catalyze the formation of XAN dependent radicals leading to degradation of XAN as well as to radical processes with the detector reagent and thus to false positive results (Schempp et al. 2010). As a first consequence of this investigation, XAN cannot longer be classified as a radical scavenger, but as an inductor of radical producing pathways. This was also supported by very recent *in vitro* data from cellular test systems (Strathmann et al. 2010). These handling difficulties prompted us to further investigate the behaviour of XAN in cell culture to give helpful suggestions for the reliable design of cellular experiments with XAN. In our investigations, we first determined the solubility of XAN in cell culture medium (Dulbeccos Minimum Essential Medium, DMEM) containing different concentrations of FCS. In a second step, the recovery of XAN from medium was determined as a function of FCS concentration. Additionally, the recovery of XAN from medium supplemented with 10% FCS was measured using different plastic materials routinely applied in cell culture like Petri dishes, cell culture flasks, falcons and 96-well plates. Likewise, the feasible conversion of XAN to isoxanthohumol was investigated. As fluorescence microscopy, especially fluorescence imaging in living cells, is a tool with increasing importance for cellular studies we also analyzed the fluorescence behaviour of XAN in a cellular system at different concentrations of FCS.

Table: Solubility of XAN in DMEM after 3 and 24 h (37 °C, 5% CO_2, falcon tubes), and effect of FCS supplementation.

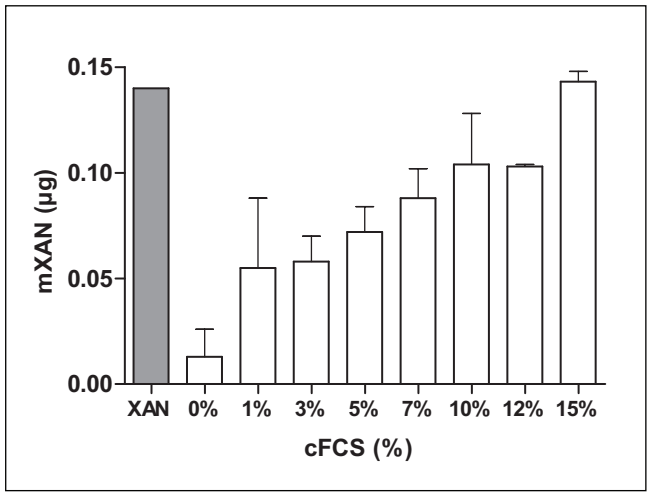
Medium	Incubation time (h)	Solubility (mg/mL)
DMEM	3	< 0.05
	24	< 0.05
DMEM + 1% FCS	3	2
	24	4
DMEM + 5% FCS	3	5
	24	10
DMEM + 10% FCS	3	16
	24	24

2. Investigations, results and discussion

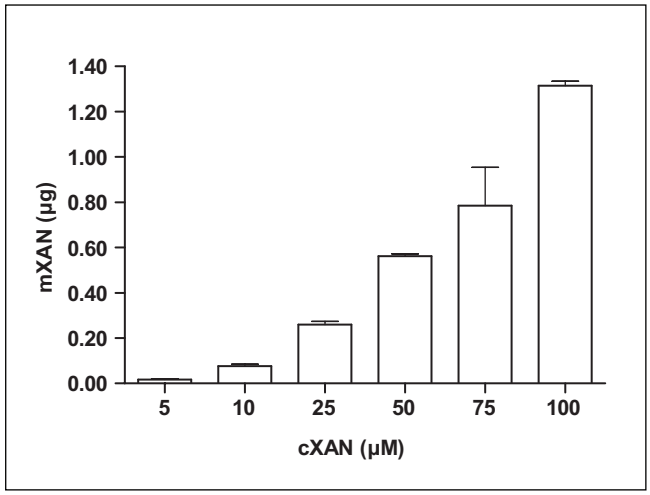
The solubility tests for XAN in pure DMEM, according to the standard method of Li et al. (1998) resulted in a solubility of less than 0.05 mg/L (3 h, 37 °C, centrifugation and filtration). This is in consideration of the moderately different experimental design in the same range as determined by Stevens et al. (1999) with 1.3 mg/L (water, 4.5 h, 23 °C and centrifugation), underlining again the bad solubility of XAN in aqueous medium. Addition of FCS resulted in an increase of solubility to 2, 5 and 16 mg/L for DMEM containing 1, 5 and 10% FCS (after 3 h). As assays often required a longer incubation time, XAN solubility was also determined after 24 h (37 °C) leading to an increased solubility of 4, 10 and 24 mg/L, respectively (Table). Accordingly, the maximal solubility of XAN under cell culture conditions for 3–24 h (37 °C, 10% FCS) can be estimated to be 50–75 μmol/l. As a consequence, the application of test concentrations clearly higher than 75 μM XAN (Miranda et al. 1999; Vogel et al. 2008) renders the results somewhat uncertain as the accurate concentration of XAN in the assay is not exactly determinable due to a clear exceeding of the solubility. In such cellular systems, an equilibration between solved, unsolved and intracellular XAN can be expected qualifying for example a determined IC_{50} value of a cytotoxicity assay.

To verify the effective XAN concentration in an assay a recovery experiment was performed by subjecting 10 μM XAN to DMEM supplemented with different FCS concentrations (1–15%). After 3 h incubation at 37 °C (in Falcon tubes) XAN was extracted from the medium and quantified by HPLC. As expected, results showed that the measured XAN concentration is a function of the percental amount of FCS in medium (Fig. 2A). Surprisingly, the measured XAN concentration is significantly lower in comparison to the inserted 10 μM even in FCS concentrations sufficient for an entire dissolution of the compound. A series of recovery experiments with XAN concentrations of 5, 10, 25, 50, 75 and 100 μM (3 h, 37 °C, 10% FCS) showed that the reduced amount of recovered XAN is not due to a reduced solubility as higher concentrations of dissolved XAN can be easily obtained (Fig. 2B). We therefore concluded that most likely also absorption phenomena on plastic material can be obtained for XAN under normal assay conditions. This was pursued by incubating 10 μM XAN (3 h, 37 °C) in DMEM supplemented with 1, 5 and 10% FCS in different cell culture material like Petri dishes, flasks, Falcon tubes and 96 well plates from different suppliers (Fig. 2C). Experiments revealed remarkable differences concerning the recovery of XAN from the medium depending on the used cell culture material. For all materials, a satisfying recovery (> 90%) of XAN requires a minimum of 10% FCS in the medium, whereas 1 or 5% FCS resulted in a severe loss (~50% or more) of the test compound (Fig. 2C).

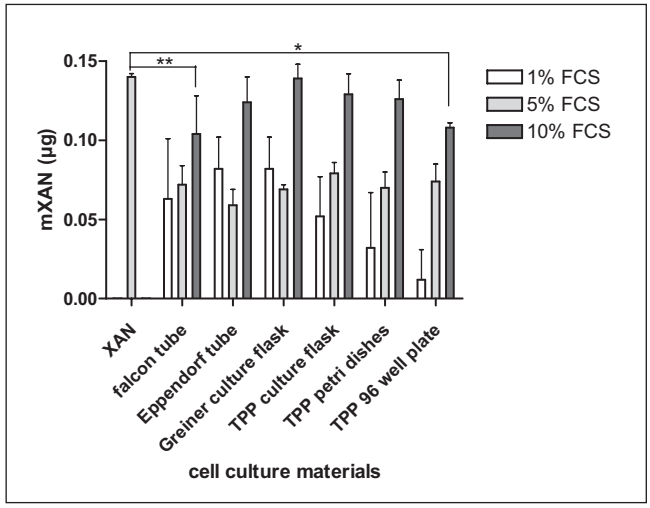
On the other hand, high FCS concentrations can also hamper results from cellular *in vitro* experiments, as the fluorescence



(A)



(B)



(C)

Fig. 2: **A**. Recovery of XAN (10 μM) after incubation (3 h, 37 °C) in DMEM supplemented with different concentrations of FCS (1–15%) in Falcon tubes (n = 4). **B.** Recovery of XAN from different concentrations (5, 10, 25, 50, 75 and 100 μM) incubated in DMEM supplemented with 10% FCS (n = 4). **C.** Recovery of XAN after incubation of 10 μM XAN (3 h, 37 °C) in DMEM supplemented with 1, 5 and 10% FCS in different cell culture material like Petri dishes, flasks, Falcon tubes and 96 well plates from different suppliers (n = 3). All values in comparison to control (XAN)

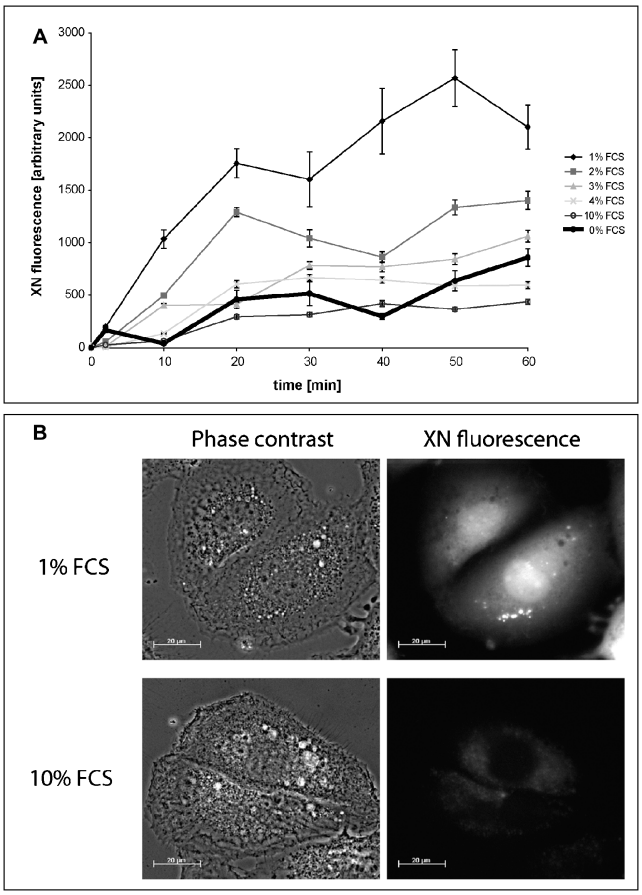


Fig. 3: Measurement of the time-dependent increase of XAN fluorescence in human hepatic stellate cells by fluorescence microscopy. **A** XAN fluorescence in hepatic stellate cells incubated with 10 μM XAN in DMEM containg 1, 2, 3, 4, 10% or no FCS over a period of 60 min. Intensity values for all timepoints after XAN addition were corrected for background fluorescence present at time point 0. Data points represent means of multiple cells ± SD. **B** XAN fluorescence in hepatic stellate cells after 60 min incubation with 10 μM XAN in DMEM with 1% (upper images) and 10% FCS (lower images). The weak fluorescence present in cells incubated with 10% FCS in medium is mostly autofluorescence, which is also present in cells that received no XAN (not shown). Scale bars: 20 μm

properties of substances can be affected. As shown by Wolff et al. (2011) the cellular intake of XAN into cells can be followed by imaging of XAN fluorescence. In our experiments, we followed the time dependent cellular intake of 10 μM XAN in immortalised human hepatic stellate cells (Schnabl et al. 2002) with fluorescence microscopy (Fig. 3A) varying the FCS concentration from 1–10%. Results showed that a reasonable increase was only observable with 1% FCS in medium. At higher FCS concentrations XAN fluorescence inside the cells was impaired, probably due to quenching or a spectral shift. In cells incubated with 10% FCS in culture medium hardly any XAN fluorescence was visible (Fig. 3B) even though it could be detected and quantified via HPLC, as shown by Wolff et al. (2011).

Likewise, the complete omission of FCS impaired the detectable XAN fluorescence inside the cells. This may be caused by different factors. On the one hand, increased absorption to the culture dish or decreased solubility of XAN could alter the available amount of XAN to be taken up by the cells. On the other hand, binding of XAN at least to some proteins contained in the FCS could be a premise for its unimpaired uptake into the cells.

The reported conversion of chalcones to flavanones prompted us to look also for the appearance of isoxanthohumol besides XAN under the above mentioned cell culture conditions. This reaction is not observable after 3 h (DMEM, 10% FCS, 37 °C), whereas the longer incubation time (24 h) resulted in the generation of isoxanthohumol even in non-cellular conditions (data not shown).

Experiments on the solubility of XAN in FCS supplemented DMEM showed that under normal cell culture conditions the solved concentration is in the range of 50–75 μM. The concentration of solved XAN is further reduced by its absorption to plastic material. The recovery experiments clearly revealed that minimally 10% FCS in medium is necessary to reduce the observed absorption to the plastic to a tolerable value. In contrast, FCS concentrations higher than 1% hampered fluorescence measurements with XAN rigorously. As a general conclusion, we suppose that for several lipophilic natural products a limited solubility under assay conditions, absorption to plastic material and a possible interaction with FCS in cellular *in vitro* experiments can be assumed. A routine determination of the maximal solubility and stability especially with regard to the used FCS concentration seems to be an appropriate parameter for the generation of reliable *in vitro* results.

3. Experimental

XAN was provided by NATECO$_2$ (Wolnzach) with a purity > 98% (determined by HPLC as described as stated below. For determination of XAN solubility and recovery, the same method was applied. All solvents (MeOH/H_2O) used were of HPLC quality. DMEM (Gibco, Invitrogen) was supplemented with different concentrations (1–15%) of FCS (Biochrom AG Berlin, Germany) for the solubility and recovery experiments. For recovery experiments, stock solutions of XAN were added to the medium and incubated in Petri dishes d 100 × 20 mm, 96 well plates, culture flasks 75 cm^2 (all from TPP, Trasadingen, Switzerland), culture flasks 25 cm^2 (Greiner bio-one, Frickenhausen, Germany), tubes 1.5 ml and 2 ml (Eppendorf, Hamburg, Germany) and centrifuge tubes 15 ml (MEUS s.r.l., Piove di Sacco, Italy) for 3 or 24 h (37 °C, 5% CO_2, humidified incubator). After addition of two volumes (v/v) of acetone, the samples were vortexed and centrifuged at 20,000 g (4 °C). To purify the samples, the supernatants were centrifuged through NanoSep centrifugal devices (Cat. 5168502 VWR, Darmstadt, Germany) at 20,000 g (4 °C).

The analytical HPLC system consists of an Elite La Chrom system (VWR-Hitachi, Germany) equipped with a L-2455 diode array detector, a thermostated L-2200 autosampler, a L-2130 pump and a L-2350 column oven. A Purospher® Star RP-18e (column size: 250 x 4.0, particle size: 5 μm, Merck, Darmstadt, Germany) was used as column. Detection wavelength was 368 nm. The eluents were water/0.1% formic acid (A) and acetonitrile/water (95/5, B). The injection volume of the samples was 20 μl and the following elution-program was used with a flow rate of 1.0 ml per min (at 30 °C): 0–9 min 57.8%–68.2% B, 9–14 min followed by equilibration to 57.8%.

Solubility of XAN was determined according to the method of Li et al. (1998, modified). Shortly, 1 mg XAN was continuously vortexed for 3 h or 24 h with 100 μl DMEM containing 1%, 5% or 10% FCS. Subsequently, 300 μl were filtered through a 0.45 μm Acrodisc® 13 mm syringe filter (PALL, Ann Arbor, MI USA) and then extracted as described above.

Fluorescence imaging experiments were performed using a computer-controlled inverted research microscope (Carl Zeiss Cell Observer), consisting of a Zeiss Axio Observer (Carl Zeiss, Göttingen, Germany) with Software AxioVision 4.8.1 (Carl Zeiss MicroImaging, Germany), following the protocol published by Wolff et al. (2011). For imaging of XAN, a custom-built filter set was used (460/80 FT 475 BP605/40). Quantification of cellular XAN fluorescence at individual timepoints was done automatically by the ASSAYbuilder Physiology Analyst software (Carl Zeiss MicroImaging, Germany), using a phase contrast image to determine the cell boundaries.

Values are presented as mean ± SD as indicated (with n = 3 or 4). Comparison between groups was made using ANOVA, followed by Tukey's Multiple Comparison Test as post test. A p value < 0.05 was considered statistically significant. For elimination of outliers, the test according to Grubbs was applied. All calculations were performed using GraphPad.

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