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Optimizing Growth Conditions for Digoxin Production in *Digitalis lanata* Ehrh

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ABSTRACT

Objective: Digoxin is a therapeutic cardenolide widely used to treat various heart conditions such as atrial flutter, atrial fibrillation and heart failure in both Western as well as Chinese medicine. Digoxin is extracted from cultivated *Digitalis lanata* Ehrh. plants, known as Mao Hua Yang Di Huang in Chinese medicine. This manuscript presents two studies that were conducted to optimize the cultivation conditions for digoxin production in the TCM Mao Hua Yan Di Huang in a greenhouse under GAP conditions.
Methods: Two experiments were designed in which 4 growth conditions were compared. Levels of digoxin, gitoxin, digitoxin, α-acetyl-digoxin, β-acetyl-digoxin were measured using HPLC-UV and compared between the conditions.
Results: Normal soil, no CO_2 enrichment combined with a cold shock was found to be the optimal condition for producing digoxin in the first experiment. Gitoxin content was significantly lower in plants grown in this condition. Mechanical stress as well as the time of harvesting showed no statistically significant differences in the production of cardenolides. In the second experiment the optimal condition was found to be a combination of cold nights, sun screen, fertilizer use and no milled soil.
Conclusion: This study shows that digoxin production can be increased by controlling the growth conditions of *D. lanata* Ehrh. The effect of cold was important in both experiments for improving digoxin production. Cultivating Chinese herbal medicines in optimized greenhouse conditions might be an economically attractive alternative to regular open air cultivation.
Key words: *Digitalis lanata* Ehrh., mao hua yang di huang, cardenolides, content optimisation, TCM
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INTRODUCTION

Digitalis cardenolides have been used in both Western as well as Chinese medicine to treat various heart conditions. In Western medicine the cardenolide digoxin is mostly used to treat atrial flutter, atrial fibrillation and heart failure. In Chinese medicine Mao Hua Yang Di Huang is used for congestive heart failure, angina, myocardial infarctions and cardiac insufficiency[28], mainly in Shanghai, Zhejiang province of China and Europe[27]. Digoxin is prescribed 20 million times a year in the US only, is well tolerated by patients, although it is currently under suspicion to increase mortality when used in the treatment of atrial fibrillation[25]. Digoxin improves cardiac output, improves the neurohormonal profile and decreases sympathetic activity in heart failure patients[9]. Since digoxin production is relatively inexpensive, is safe to use and has beneficial effects, it is an important natural product in cardiology in both Western world as well as in Chinese medicine.

There are several examples of drugs developed from isolated compounds from Traditional Chinese Medicines. Dr. Tu You You received the Nobel Prize in 2015 for isolating Artemisinine, a cure for Malaria, from *Artemisia annua*, which has saved millions of lives. Prof Zhu Chen identified Arsenic trioxide in a well-known Chinese medicine which was shown to be very effective in treating acute promyelocytic leukemia (APL)[26]. In this paper two experiments for improving the isolation of digoxin from Mao Hua Yang Di Huang, known as *Digitalis lanata* Ehrh., are discussed.

Therapeutic cardenolides are obtained from two *Digitalis* species, *D. lanata* and *D. purpurea*. Both primary glycosides such as the lanatosides and secondary glycosides such as digoxin are used. The compounds are extracted from plants cultured in specific conditions to optimize the production of these cardenolides. In this paper an overview is presented of the scientific literature concerning conditions that can improve the production of cardenolides and in particular digoxin and its precursor lanatoside C. Based on this review, an experiment was conducted in which the effects of 4 growth conditions on the cardenolide production in *D. lanata* Ehrh. were examined. Subsequently, a second study was conducted to optimize those conditions based on the results of the first study.

1. Biology of cardenolide production

Cardenolides are synthesized from steroids such as cholesterol via what is considered 'a complex multidimensional metabolic grid' involving various pathways[16]. The first cardenolide specific step is the 5β-reduction of progesterone to 5β-pregnane-3,20-dione by the enzyme progesterone 5β-reductase (5β-POR). The next crucial reaction is the 14β-hydroxylation. However, Kreis and others offer evidence that another route of cardenolide genin formation is operative in *D. lanata,* leading to fucose-type cardenolides that are not used in therapy. Down regulating this fucose pathway might improve the production of the pharmaceutically relevant digitoxose-type cardenolides in *D. lanata*[5,14].

Some research has been conducted on the precursors of the cardenolides in *Digitalis* species. Cholesterol, which is a major sterol in *Digitalis* species, is considered the main precursor. It was found in *D. purpurea* that pregnenolone was the main biotransformation product of cholesterol. Other compounds that might serve as cardenolide precursors are β-sitosterol, smilagenin and sodium glycocholate[14].

Pregnenolone is considered the starting point of the cardenolide pathway and is incorporated in digitoxigenin and to a lesser extend in digoxigenin and gitoxigenin. The cardenolide content of light-grown *D. lanata* shoot cultures can be increased by feeding the plants: 21-hydroxypregnenolone (161%), 5β-pregnane-3,20-dione (240%), 5β-pregnan-3β-ol-20-one (30%), 5β-pregnane-3β,14β,21-triol-20-one (430%), 23-nor-5,20 (22)E-choladienic acid-3β-ol (80%). However, feeding the plants pregnenolone, progesterone, cortexone, 5α-pregnanes, 5β-pregnan-21-ols, and 23-nor-5-cholenic acid-3ξ,2O-diol had no effect on cardenolide content[14].

The lanatosides A and C, the major cardenolides of the *Digitalis* leaves, were found to be the major compounds in the mesophyll protoplasts and the vacuoles isolated from them. On the other hand, the secondary glycosides digitoxin, α-acetyldigitoxin and α-acetyldigoxin could only be detected in the leaf extracts, not in other plant parts[15].

The primary glycosides entering the mesophyll cells or produced in the cells are trapped in vacuoles. By wounding the leaves these primary glycosides are freed from the vacuoles and can be quickly metabolized by the membrane bound Cardenolide 16′-O-glucohydrolase I (CGHI) into the toxic secondary glycosides.

2. Factors influencing cardenolide production

Table 1 summarizes the effect of several factors on the cardenolide content of various *Digitalis* species described in the literature. The results are presented in three main topics: 1) experiments in which the cultures are fed with a compound, 2) the effect of stress factors, 3) the effect of light, darkness, and other factors. In the following part of the report these findings will be discussed.

2.1 The influence of compound feeding on cardenolide production

Malonate was found to be the most efficient and effective precursor in 5β-cardenolide synthesis[10]. This effect was measured in a number of cardenolides including lanatoside C and digoxin. Cardenolide composition was more stable in water cultured greenhouse plants than soil cultured greenhouse plants.

Gärtner and others found that feeding *D. purpurea* with cholesterol or pregnenolone did not stimulate the accumulation of cardenolides. 5β-pregnan-3,20-dione however did increase cardenolide accumulation by 200-300% according to a personal communication by Stuhlemmer referred to in several papers[7,22]. Progesterone has been reported by one author to have a cardenolide stimulating effect of 180%[13], but other authors disagree with these findings[14,16].

The cardenolide content of *D. lanata* shoots cultured in liquid medium in light conditions increased by 161%, 240%, 30%, 430% and 80% when 100mg/L of 21-hydroxypregnenolone, 5β-pregnane-3,20-dione, 5β-pregnan-3β-ol-20-one, 5β-pregnane-3β,14β,21 -triol-20-one, 23-nor-5,20 (22)E-choladienic acid-3β-ol, respectively, were administered. These compounds were for 70% consumed to produce fucose-type cardenolides and for 30% to produce digitoxose-type cardenolides. Both pathways were stimulated equally well by the compounds mentioned[14].

Shoot-forming cultures of *Digitalis purpurea* L. were grown in various modifications of Murashige-Skoog medium to test the effects of vitamins, natural extracts, plant growth substances, carbon and nitrogen sources and phosphate on their growth and digitoxin formation[11]. Sucrose, glucose and raffinose were suitable carbon sources for both growth and digitoxin formation. The optimum concentration of sucrose was 3%. Reduction of the basal nitrogen concentration by one-third increased the digitoxin content per unit weight without suppression of growth. The optimal ratio of nitrate-nitrogen to ammonium-nitrogen was 2.

A threefold increase in the phosphate concentration (510 mg/l KH_2PO_4) improved both growth and digitoxin content. Thiamine HCl and myo-inositol were required for digitoxin formation, whereas glycine, nicotinic acid and pyridoxine HCl, usual components of the medium, were not required for either growth or digitoxin formation during the three passages examined. However this effect of phosphate on cardenolide production was not found in *D. obscura* shoot cultures[8]. Coconut milk improved growth with no reduction in the digitoxin content per unit weight. Giberellic and abscisic acids at 0.01 to 0.1 mg per liter slightly improved digitoxin formation. Kinetin had no clear positive effect on either growth or digitoxin formation[16-17].

In another study Hagimori and colleagues explored the optimum concentrations of some compounds for the accumulation of digitoxin in green, light-grown, shoot-forming cultures of *D. purpurea* in Murashige-Skoog medium. The optimal concentrations and compounds were: benzyladenine, 0.01 to 1 mg/l, indoleacetic acid, 0.1 to 1 mg/l, anaphthaleneacetic acid; 0.1 mg/l, and 2,4-dichlorophenoxyacetic acid, 0.01 mg/l[11-12].

Roca-Pérez and colleagues studied the relationships between soil and leave micronutrients on cardenolide production in 10 natural populations *D. obscura* plants. Zinc and

Table 1. Factors influencing cardenolide production

Factor	Target	Species	Effect	Author
Feeding				
malonate feeding	5β-cardenolides	3-4 m old D. lanata on water	most effective precursor of 5β-cardenolide synthesis	Groeneveld 1992
cholesterol feeding	cardenolides	D. purpurea shoot culture	no effect on cardenolide synthesis	Gartner 1993
pregnenolone feeding			no effect on cardenolide synthesis	Stuhlemmer
5β-pregnan-3,20-dione	cardenolides	D. purpurea shoot culture	200-300% increase in cardenolide accumulation	
precursor feeding	cardenolides	D. lanata plants	The cardenolide content of light-grown shoot cultures increased by 161%, 240%, 30%, 430% and 80% when 100mg/L of 21-hydroxypregnenolone, 5β-pregnane-3,20-dione, 5β-pregnan-3β-ol-20-one, 5β-pregnane-3β,14β,21-triol-20-one, 23-nor-5,20 (22)E-choladienic acid-3β-ol, respectively, were administered.	Haussmann 1997
compounds in medium	digitoxin	D. purpurea shoot culture	Optimal concentrations: benzyladenine, 0.01 to 1 milligram per liter, indoleacetic acid, 0.1 to 1 milligram per liter, anaphthaleneacetic acid; 0.1 milligram per liter, and 2,4-dichlorophenoxyacetic acid, 0.01 milligram per liter.	Hagimori 1982a
carbohydrate feeding	digitoxin	D. purpurea shoot culture	Highest digitoxin content with sucrose, glucose or raffinose feeding.	Hagimori 1982b
nitrate			Best nitrate concentration was 1,100 mg/liter NH_4NO_3 + 1,267 mg/liter KNO_3. Best nitrate-nitrogen to ammonium-nitrogen ratio was two to one.	
phosphate			Highest digitoxin yield with 510 mg/l phosphate.	
KH2PO4 & MnSO4	cardenolides	D. obscura shoot culture	No effect of phosphate and manganese on cardenolide production.	Gavidia 1997
Leave micronutrients	cardenolides	D. obscura populations	Zn and Mn are negatively correlated with cardenolide production. Fe is positively correlated.	Roca-Pérez 2004
Stress				
Wounding	P5βR RNA	2 m old D. purpurea on soil	P5βR RNA levels remained constant	Pérez-Bermúdez 2010
Cold shock			P5βR RNA levels remained constant	
Heat shock			P5βR RNA levels remained constant	
NaCL			P5βR RNA levels remained constant	
Wounding	P5βR2 RNA	2 m old D. purpurea on soil	max after 1 hour, then sharp decrease. After 15 hours second max	Pérez-Bermúdez 2010
Cold shock 4°C			max upregulation at 2 hours	
Heat shock 41°C			max at 1 and 2 hours	
NaCL			After 48 h maximum upregulation	
ACC			Upregulation	
H2O2			Upregulation	
methyl jasmonate			no effect	
salicylic acid			no effect	
Wounding	evatromonoside	2 m old D. purpurea on soil	max increase after 4 hours	Pérez-Bermúdez 2010

Table 1. (Continued)

Factor	Target	Species	Effect	Author
Wounding calcium depletion	digitoxin cardenolides	*D. thapsi* undifferentiated cultures	diminished Cardenolide production increased and extracellular H_2O_2 increased. H_2O_2 and superoxide dismutase stimulate cardenolide production.	Paranhos 1999
CO_2 and water stress	cardenolides	*D. lanata* plants	CO_2 enrichment (1000 ppm) increased biomass and cardenolide content to about 60%. Digoxin increased while two othercardenolides decreased. Water stress reduced plant weight, digoxin anddigitoxigenin. CO_2 enrichment reduced the water stress effect.	Stuhlfauth 1987
inhibitor feeding	cardenolides	*D. lanata* embryogenic tissue culture	0.3 mM glutathione biosynthesis inhibitor buthionine-sulfoximine (BSO) increased the digitoxin yield to 183%. 3.3 mM Glutathione (GSH) reduced the cardenolide yield to 6 %. The effects are related to tissue differentiation.	Berglund 1993
Light & Darkness light/darkness	digoxin	*D. lanata* plants	16 h photoperiod, in 300 Em2s light intensity, digoxinincreasing phase is shortest (6 to 7 weeks). High light intensity increases the variation in digoxin content. Decrease in daylight duration decreases digoxin contents.	Brugidou 1988
light/darkness	cardenolides	*D. lanata* shoot culture in liquid medium	Tissue differentiation is essential for cardenolide production. 0,6 μmol/g cardenolide production in continuous light conditions. In dark conditions cardenolide content gradually decreases. DAT,CGH I and LAE are downregulated in dark conditions, DGT is unaffected.	Eisenbeiß 1999
season	cardenolides	*D. obscura* populations	The lowest production was recorded in May, followed by a fast cardenolide accumulation in summer, a decreasing phase in autumn, and a stationary phase in winter. Dop5βr gene (progesterone 5β-reductase) expression increased from February to July and a reduction in autumn. Positive correlation between biomass and cardenolide content.	Roca-Pérez 2004b
Other Age (12 m & 18 m)	cardenolides	*D. lanata* plants	18 m plants higher lanatosid C and digoxin content, but lower lanatosid A & B content than 12 m plants.	Braga 1997

manganese concentrations in the leaves are found to be negatively correlated with cardenolide production while iron is positively correlated. Manganese added to the culture medium of *D. obscura* shoot cultures was found to have no effect on cardenolide production[8].

2.2 The influence of stress on cardenolide production

Digitalis species use cardenolides to defend against herbivores. Wounding of the leaves induces the release of cardenolides. Here the effects of various types of stress factors such as wounding, heat and cold on cardenolide production are discussed.

A recent study focused on the effect of stress conditions on the expression of P5βR and P5βR2 in *D. purpurea*, genes encoding for the first cardenolide pathway specific enzyme progesterone-5β-reductase. *D. purpurea* seeds were grown in a glasshouse on soil mixed with vermiculite, 22°C, 16 hours of light. The following stress factors were applied to two month old plants: 1) wounding by making 1mm holes across the lamina of the leaves, 2) cold stress by exposing the plants to 4°C up to 4 days, 3) heat stress by growing the plants at 41°C for up to 4 hours, 4) salt stress was applied by watering the plants with 250 mM NaCl, 5) chemical treatment by spraying with methyl jasmonate, salicylic acid, 1-aminocyclopropane-1-carboxylic acid and hydrogen peroxide and collection of leaves at different time points up to 48 hours[19].

The results show that none of the stress factors increases or decreases the expression of P5βR. However they do affect the expression of P5βR2. One hour after wounding a maximum expression of P5βR2 is seen. Cold stress induces a maximum up regulation at 2 hours, heat stress at 1 and 2 hours. Watering with salty water induces a maximum expression at 48 hours. Of the chemical stressors 1-aminocyclopropane-1-carboxylic acid and hydrogen peroxide increased the expression of P5βR2, methyl jasmonate and salicylic acid had no effect.

Wounding also increased cardenolide production with a maximum after 4 hours mainly caused by an increase in evatromonoside. The concentration of digitoxin decreased a little. The authors suggest that wounding induces an increase in the amount of less polar cardenolides and a decrease of the polar cardenolides. Less polar, more lipophilic, compounds can more easily pass membranes indicating that wounding might increase the toxicity of the plants through this mechanism.

Paranhos and others show that cardenolide production can be increased in *D. thapsi* cell cultures by depriving the cells of calcium. The production is still negligible compared to that in whole plants, but the mechanism might be of interest. Calcium depletion was found to increase the H_2O_2 concentration which might act as a second messenger in cardenolide synthesis[18]. However Berglund points out in a review that cytosolic calcium is a requisite for the production of secondary metabolites in response to stress.

Cytosolic calcium can be regulated by light, growth factors like abscisic acid, gibberellins and auxins[2]. Research in the area of plant defence mechanism in general shows that H_2O_2 increases the production of secondary metabolites in plants. UV-light and ozone can increase H_2O_2 production and induce secondary metabolism although this is not specifically studied in *Digitalis* species. Berglund did study the effect of the glutathione inhibitor BDO, which increases H_2O_2, on an embryonic tissue culture strain of *D. lanata* and found an increase of digitoxin of 183%[1].

The effect of CO_2 enrichment to 1000 ppm was found to increase fresh and dry weight of *D. lanata* plants by about 60%. It also increased the cardenolide content with 60%, which was therefore directly related to the increase in growth. The digoxin content increased 10% per gram dry weight in enriched plants. Moderate water stress reduces the weight pf the plants and especially reduces digitoxigenin content. The authors suggest that CO_2 fertilization is promising for the cultivation of medicinal plants such as *D. lanata*[23].

2.3 The influence of light, season and geography on cardenolide production

Brugidou and others compared *D. lanata* plants grown in natural light conditions with plants grown in artificial light conditions. The artificial conditions were 300 µE/m^{2}s, 22°C, 60% humidity and 16 light hours. In these conditions digoxin increased rapidly and became stable at the 'dense rosette' morphological stage. In natural light conditions from November to March digoxin contents increased less rapidly and reached lower concentrations than in the artificial situation. The 'dense rosette' stage was never reached. From May to October a fast increase phase of digoxin was observed but from August to September this increase was less[4].

Another study shows that cardenolide production decreases when plants are moved to darkness. The enzymes DAT, CGHI and LAE are downregulated in dark conditions while DGT is unaffected. Cardenolides were still produced in dark conditions. Intact chloroplasts were not needed but tissue differentiation was found to be most essential for cardenolide production[6].

The effect of the season on cardenolide production was studied in *D. obscura* leaves taken from the intermediate region of the shoots. The lowest cardenolide production was recorded in May, followed by a fast cardenolide accumulation in summer. Cardenolide contents decreased again in autumn and remained constant in winter. The expression of the progesterone-5β-reductase gene showed a similar pattern, an increase from February to July followed by a reduction in autumn[20-21].

A large variation of cardenolide production was found between populations of *D. obscura* grown in different environmental conditions which could be as large as 70%. Within population variation was reported to be around 20%. This observation shows that environmental conditions have a large effect on cardenolide production. Biomass was found to be positively correlated with cardenolide production. Braga reported a similar large variation between cardenolide

Table 2.   Growth conditions in Experiment 1

Conditions	T	Water	CO$_2$	Dry ground
1	-		No	No
2	Cold & Heat shock		Yes	No
3	-	Salt stress	No	Yes
4	Cold shock		No	No

contents of *D. lanata* populations in Brazil. Additionally cardenolide concentration was found to be larger in 12 month old plants than in 18 month old plants. However the concentration of lanatoside C and digoxin was larger in the 18 month old plants and was therefore a larger percentage of the total cardenolides in the 18 month old plants[3].

In this study, experiments were designed based on the above literature findings as well as pragmatic reasons. The main aim of the study was to discover factors that improve cardenolide production in *D. lanata* Ehrh. that can be cost-effectively implemented in greenhouse conditions. The factors that promote digoxin production are divided in three groups, precursor feeding, stressors and light conditions. The first group, precursor feeding, was deemed too costly to implement. Cold, heat, salt stressors and CO$_2$ were chosen from the second category for the first experiment. In addition, the time of harvesting was an important study factor. The conditions of the second experiment were based on the results of the first experiment.

MATERIALS AND METHODS

Both studies were carried out in a greenhouse on private land in South Drenthe (52.74571°N 7.00270°E, at 14 m elevation), after permission was obtained from the owner. The climate in this area of the Netherlands is a mild sea-climate with average temperature 2 degrees below the national temperatures. The greenhouse was heated to a minimum temperature of 15°C and ventilated at 25°C in both experiments.

1. Experiment 1
In the first experiment, *D. lanata* Ehrh. is grown in four different culturing conditions in order to test which of these conditions leads to the largest amount of digoxin and α-acetyldigoxin (a-a-digoxin) production by the plants (see table 2). At the same time, low concentrations of β-acetyldigoxin (b-a-digoxin), gitoxin and digitoxin are preferred. In addition, the plants are harvested at different time points to test the effect of growth duration on digoxin levels.

The conditions were realized in the following manner. To give the plants a cold shock, the air windows are opened 100% during the coldest moment of the night, which is usually just before dawn. A climate computer controls the drop in temperature. The heat shock was obtained by closing the air windows at the hottest moment of the day, using a climate control computer. The dry ground was realized by reducing the amount of water given. A tencio meter was used to control

the water percentage of the top layer of the soil. CO2 was introduced into the growth chamber by a petrol heater, which rapidly increases the amount of CO2 in the chamber. This was done two times a day, once in the morning and once in the afternoon. Salt stress was induced by increasing the amount of salt in the water from an EC of 1.5 to 2.5.

1.1 Study design
D. lanata seeds were sown on the 20th of February 2012 in neutral topsoil. *D. lanata* plants were harvested at a number of time points in 2012: 13th of September, 27th of September, 4th of October, 18th of October, 25th of October and an additional three harvests were conducted in June, July and October. At the 18th of October there was an extra harvest of plants of each condition which were subjected to the 'Tuch' condition in addition to the regular condition. The 'Tuch' condition consists of dragging a cloth over the plants to cause mechanical stress from movement of the leaves. This was done two times a day, early in the morning and late in the afternoon.

At each time point over ground parts of 20 plants, randomly chosen, were harvested for each of the four conditions. These 20 plants were then carefully separated into single leaves and stems with scissors. This careful separation is critical to avoid decompartimentation within the cell tissue. The separated plant parts were then dried in shade with (30 ± 5)°C in one layer to avoid humidity nests, until they were fracturable. The dried samples were then homogenised and milled, 320mg of material was weighed and prepared for HPLC analysis. This results in 9 samples for each of the 4 conditions.

1.2 HPLC measurements
1.2.1 Sample preparation   Solutions to measure the plant samples: about 4.0 g fine milled homogenized dried leaves are weighed into a ultraturrax dispenser tube, 5.0 g bi-destillated water added, intensively mixed, covered with para film and then stored for 18 h for fermentation at 35 °C. Then a solution of water/ethanol/Lead (II) acetate solution (V/V/V) is pipetted into the fermented solution. Subsequently this solution is homogenized with ultraturrax (2000 U/min) and extracted in an ultrasonic bath for 15 min with agitating it several times. Then, the sample is transferred into a centrifuge glass which is then covered with para film and centrifuged for 5 min (5000 U/min). 40.00 ml of the clear solution are pipetted into another centrifuge glass, 40.00 ml of the Na$_2$HPO$_4$ solution, stirred, the centrifuge glass covered with para film and the precipitation centrifuged for 5 min (5000 U/min). 20.00 ml of the clear solution is pipetted into a 50 ml volumetric flask and adjusted to 50 ml with solvent mixture.

Solutions for testing the system: about 5 mg each digitoxigenin, digoxigenin-mono-digitoxosid, diginatin, digoxigenin-bis-digitoxosid, neodigoxin, lanatoside C, digoxin, digitoxigenin-tetrakis-digitoxosid, α-acetyl-digoxin, β-acetyl-digoxin, gitoxin, and digitoxin are weighed into a 100 ml volumetric flask, solved in methanol and adjusted with methanol to

Table 3. HPLC gradient

Time (min)	Eluent I	Eluent II
0	90	10
3·5	75	25
8	25	75

exactly 100 ml (solution A). About 50 mg digoxin are weighed into a 100 ml volumetric flask, solved in 45.00 ml methanol, 5.00 ml solution A and 45.00 ml SM added and after tempering to room conditions adjusted with methanol to exactly 100 ml (solution B).

Reference solution: about 50.0 mg digoxin is weighed into a 100 ml volumetric flask, solved in 50 ml methanol, added with 45 ml SM and after tempering to room temperature to 100 ml with methanol.

Solution for sensitivity check: 50 µL of the reference solution are pipetted into a 100 ml volumetric flask, 50 ml methanol and 45 ml SM added and, after tempering to room temperature, adjusted to 100 ml with methanol.

1.2.2 HPLC conditions   An HPLC system with UV detection (at 220 nm) was used with a Zorbax SB C 18, 50 mm X 4.6 mm, 1.8 µm column. The column temperature was held at 30 °C. The injection volume was 5 µL. A flow rate of 1.5 ml was used resulting in an analysis time of 8 minutes and an equilibration time of 4 minutes.

Acetonitrile gradient grade; methanol gradients grade; ethanol p. a.; water HPLC grade; KH_2PO_4 p.a.; Na_2HPO_4, p.a.; Na_2HPO_4, solution, saturated, about 93% g/L; Lead (II) acetate trihydrate solution 15% were used for the HPLC analysis. The following reference substances were used to identify and quantify the compounds measured in the plant samples: digitoxigenin, digoxigenin-mono-digitoxosid, diginatin, digoxigenin-bis-digitoxosid, neodigoxin, lanatoside C, digoxin, digitoxigenin-tetrakis-digitoxosid, α-acetyl-digoxin, β-acetyl-digoxin, gitoxin, digitoxin.

A gradient system was used with a mobile phase consisting of a buffer solution pH 5.5 1.0 g KH2PO4 solved in 1 L of water (see table 3). Adjustment to pH 5.5 is carried out with 1% Na_2HPO_4, p. a. solution. The organic phase consisted of 950 ml acetonitrile and 50 ml methanol. The mobile and organic phases were mixed to obtain eluent 1 (800 ml mobile and 200 ml organic phase) and eluent 2 (200 ml mobile and 800 ml organic phase). A solvent mixture (SM) contained 50 % organic phase and 50 % buffer solution at pH 5.5. Two sample chromatograms, typical retention times and molecular structures of the reference compounds are presented as supplementary material (S1, S2, S3).

1.2.3 Quantification of the compounds   Digoxin, α-acetyl-digoxin, β-acetyl-digoxin, digoxigenin, lanatoside C, digoxigenin-mono-digitoxosid, digoxigenin-bis-digitoxosid, gitoxin and digitoxin were quantified by comparing with the reference solution; all other substances are not quantified. Only those peaks of the above mentioned substances which

exceed the amounts measured in the sensitivity solution were quantified.

The following formula was used to quantify the compounds in the test samples:

$$\% \, substance = \frac{A * F * 100\,\% * 425}{m} * \left(\frac{m_{ref}}{A_{ref}}\right) * \left(\frac{P_{ref}}{100\,\%}\right)$$

A = area of the peak to be calculated in the chromatogram of the test solution

F = correction factor of the peak of the substance

m = weight (mg) of the sample

A_{ref} = area of the digoxin peak in the reference solution

M_{ref} = weight of the digoxin (mg) in the reference solution

P_{ref} = potency of the used reference substance in %

The quotient (m_{ref}/A_{ref}) of each particular weight (m_{ref}) of the reference substance and of the area of the peak of the main compound is averaged over all injections of the reference solutions of the sequence.

1.3 Data analysis

Condition effect: The mean and standard deviation of each condition over the different time points was calculated separately for each compound. Students T-test was used determine the significance between these mean values. This shows which conditions are significantly better compared to other conditions for each compound.

In addition the 'Tuch' condition on the 18th of October was compared with the 'non-Tuch' condition on the 18th of October. For this analysis the 'Tuch' values of each of the 4 conditions are used to calculate the mean and standard deviation. Then Students T-test was used to compare the mean of the 'Tuch' with the 'non-Tuch' condition for each compound.

Harvesting time effects: Individual time effects per compound and per condition were checked. Single measurements are not sufficient for statistical analysis. Therefore the response ratios of the four conditions are averaged. Standard deviations were calculated and Students T-test was used to test for significance. This results in an indication of the best time to harvest the plants without any regard to the growing condition.

2 Experiment 2

D. lanata seeds were sown on the 20th of February 2013 in neutral topsoil. In the second experiment, *D. lanata* Ehrh. was grown in eight different combinations of conditions, described in Table 4. This study was carried out on the same private land as the first experiment, after permission was obtained from the owner. Since the cold shock stressor turned out to be the most promising condition in the first experiment (see results section of experiment 1), cold was also an important factor to be further explored in this second experiment. In this experiment a combination of a cold morning and warm midday was compared with a cold night and average temperatures. Three new factors were tested on top of that, screening of the sun or not, milled soil and fertilizer use. The soil was milled with a light mill, too loosen the top 5-6 cm of the soil. The cold and heat conditions

Table 4.　Growth conditions in Experiment 2

Conditions	EC	T	Sun free	Water	Soil freezed	Fertilizer	CO$_2$	Dry ground
11	Hoog	Average	No		Yes	No	No	No
12	-	Morning cold/ midday warm	Yes		Yes	No	Yes	No
13	-	Average	No		Yes	No	No	No
14	-	Cold night	No		No	Yes	No	No
16	Hoog	Average	Yes	inline	No	No	No	Yes
17	Hoog	Average	No	overhead/ inline	Yes	No	No	No
18	Laag	Morning cold/ midday warm	Yes	overhead/ inline	Yes	No	No	No
19	Laag	Cold night	No	overhead/ inline	No	Yes	No	No

in this experiment were also realized by opening the air windows 100% during the cold period of the morning or closing them 100% during the hot period of the afternoon. Low and high EC indicate lower and higher salt concentrations in the water, low is 1.5 and high is 2.5 EC. The fertilizer consisted of calcium nitrate, magnitra, Mn-chelate, zincsulfate, borax, coppersulfate and sodium molybdate.

2.1 Study design
Each sample consisted of 20 individual plants which were picked randomly from the area by cutting the leaves about 1 cm above ground so that the underground parts were not

Table 5.　Sample weeks per condition and harvests

11	12	13	14	16	Harvest
18*	18	18*	18*	18	v
20	20	20	20	20	v
22	22	22	22	22	v
24	24	24	24	24	v
26	26	26	26	26	v
28	28	28	28	28	v
30	30	30	30	30	v
32	32	32	32	32	v

17	18	19	Harvest 1	Harvest 2	Harvest 3
25	25	25	v		
27	27	27	v		
29	29	29	v		
31	31	31	v		
32	32	32	v	v(25)**	
33	33	33	v		
34	34	34*	v	v(27)	
35	35	35*	v		
36	36	36	v	v(29)	
37	37	37	v		
38	38	38	v	v(31)	
39	39	39	v		
40	40	40	v	v(33)	
41	41*	41	v		v(25)
42	42	42	v	v(35)	
43	43	43	v		v(27)

v = weeks at which a sample was taken.
* Samples missing for HPLC analysis.
** Harvest 2 and 3 samples are taken from plants already harvested, harvest week of the earlier harvest is between brackets.

damaged. The cuttings were dried at ambient conditions in a single leaf layer in the shade. The samples were collected at several time points as outlined in Table 5. Each of the conditions 11 to 16 were harvested every other week from week 18 up to week 32 in 2013. However, samples from the plants grown in conditions 17, 18 and 19 were harvested 16 times. Furthermore, the plants in those three conditions were harvested twice at the weeks indicated with the letter v in Table 5.

The 20 individual plants, which had been taken as samples in CW 25 had been sampled 7 weeks later: CW 32 (Harvest 2), and another time in CW 41 (harvest 3). Those of CW 27 had been sampled a second time in CW 34 (Harvest 2) and a third time in CW 43 (Harvest 3). The individual plants harvested in CW 29 were harvested a second time in CW 36, those of CW 31 a second time in CW 38, those in CW 33 a second time in CW 40 and those in CW 35 in 42.

2.2 HPLC measurements
HPLC measurements of the samples were conducted with the same conditions as described in experiment 1.

2.3 Data analysis
Condition effects: Similar to experiment 1, the mean and standard deviation of each condition over the different time points was calculated separately for each compound. Students T-test was used determine the significance between these mean values. This shows which conditions are significantly better compared to other conditions for each compound.

Harvesting time effects: Individual time effects per compound and per condition were checked. Single measurements are not sufficient for statistical analysis. Therefore the response ratios of the four conditions are averaged. Standard deviations were calculated and Students T-test was used to test for significance. This results in an indication of the best time to harvest the plants without any regard to the growing condition.

RESULTS AND DISCUSSION

1 Experiment 1
Some samples were missing and some are duplicated as is shown in Table 6. In addition, the third harvest from

Table 6. Sample dates per condition

1	2	3	4
13.9.	13.9.	13.9.	13.9.
27.9.	-	27.9.	27.9.
		27.9.	
4.10.	4.10.	4.10.**	4.10.
18.10.	18.10.	18.10.	18.10.
18.10.	18.10.	18.10.	18.10.
25.10.	25.10.	25.10.	25.10.
Harvest 1	Harvest 1	Harvest 1	Harvest 1
Harvest 2	Harvest 2	Harvest 2	
	Harvest 2	Harvest 2	
Harvest 3*			Harvest 3*
			Harvest 3

* Mouldy.
** Leaves with lesions.

condition 1 was mouldy as well as the first one of the third harvest from condition 4. The 4th October harvest of condition 3 showed leaves with lesions but it was included in the analysis.

Figure 1 shows the effects of the growth condition on the measured compounds. For this figure the five harvesting dates are averaged. Condition four results in significantly higher levels of digoxin compared to the other three conditions (1 vs 4 p=0.01, 2 vs 4 p=0.002, 3 vs 4 p=0.004). Condition four also results in significantly lower levels of gitoxin

compared to condition one and three (1 vs 4 p=0.007, 3 vs 4 p=0.028). The four conditions have no significant effect on digitoxin and β-acetyl-digoxin levels in this study. The α-acetyl-digoxin levels were below the detection limit. Conditions one, two and three are not significantly different from each other for any of the metabolites. No significant differences are found between the 'Tuch' and 'non-Tuch' condition for any of the metabolites (data not shown). Data for the extra three harvests were not further analysed due to the missing data. A representative HPLC chromatogram is shown as supplementary material (S1).

The effect of growth duration on metabolite levels was evaluated as well. For this analysis the metabolite levels in the four conditions are averaged for each time point. No significant differences were found between any of the time points for any of the metabolites. The 27th of September harvesting time point however shows a trend of higher total digoxin and digoxin levels, although not statistically significant in this experiment (data not shown).

Figure 2 shows the relationships between digoxin and β-acetyl-digoxin for all conditions (four different shapes of labels) and all harvesting time points (named D1 to D5 and E1 to E3). Because all points in the figure represent single measurements, statistics are not possible. The data was mean centred to be able to compare the data of
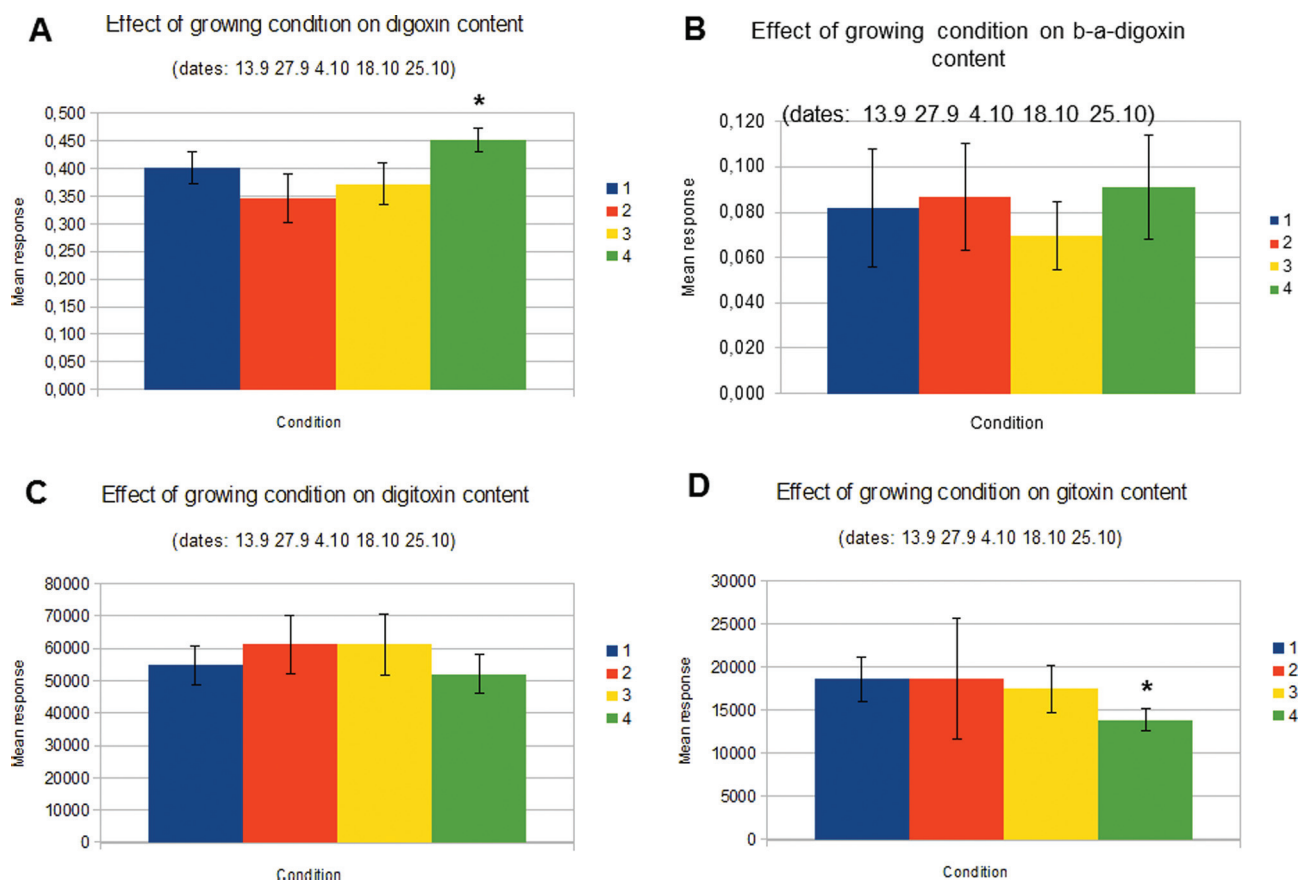
Figure 1. Effect of growing condition on *D. lanata* metabolites. Each bar (numbered 1 to 4) represents the mean value of the different time points of one compound in one condition. Standard deviations are depicted by the black error bars. Statistical significant differences are shown with an asterisk (*), p-value <0.05. Panel A shows the effect on digoxin. Panel B shows the effect on β-acetyl-digoxin, panel C on digitoxin and panel D on gitoxin.
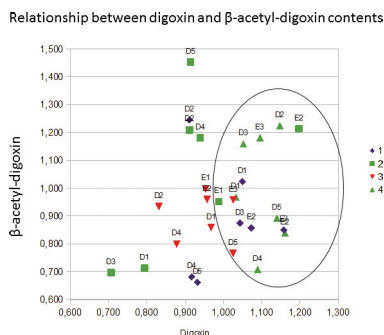
Figure 2. Relationships between digoxin and β-acetyl-digoxin levels. The conditions 1 to 4 are labelled with four different symbols and colours (the conditions are explained in table 2). Harvesting time points are labelled with D1 to D5 and E1 to E3 for the extra harvests. The condition 4 samples are indicated with a circle.

different metabolites; the actual levels of β-acetyl-digoxin are usually much lower than those of digoxin. The lower right corner of the figure contains the samples with the lowest β-acetyl-digoxin levels and the highest digoxin levels. Condition 4 samples have high levels of digoxin (they are at the right side of the figure); D4 and D5 would be optimal. Also Harvest 2 of condition 1 and Harvest 3 of condition 4 have high digoxin content compared to β-acetyl-digoxin.

From this study it can be concluded that in condition 4 the digoxin levels are significantly higher than in the other three conditions. In addition the gitoxin levels in condition 4 are significantly lower than in condition 1 and 3. Therefore, condition 4 seems the most suitable for the production of digoxin. This condition consisted of normal soil, no CO_2 enrichment and a cold shock was applied as a stressor. Secondly, it can be concluded that the 'Tuch' condition has no significant effect on the amount of digoxin, when the

measurements of the four growth conditions are averaged. Thirdly, the harvesting time effects show no statistically significant differences.

2 Experiment 2

The following samples were not available for HPLC measurements (also indicated with * in Table 4 above): week 18 of condition 11, 13 and 14; week 41 of condition 18; and weeks 34 and 35 of condition 19. Since the desired cardenolides were digoxin and a-a-digoxin, Figure 3 shows the sum amounts of those cardenolides for each harvest time point, per condition (called House in the figure). At most harvest weeks, condition 14 results in the highest amount of total digoxin. In week 32, the produce of condition 14 is slightly lower than that of condition 12. When the harvesting weeks produces of total digoxin levels are averaged per condition (see Figure 5), condition 16 is significantly lower than condition 11 (p=0,05) and 14 (p=0,02). No significant differences were found between the other condition averages of total digoxin levels.

Figure 4 illustrates the sum amount of digoxin and α-acetyl-digoxin for each harvesting week for the other three conditions (17, 18 and 19). Condition 19 seems to produce the largest amount of total digoxin. Averaging all the harvesting weeks, condition 19 produces a significantly larger amount than condition 18 (p=0.004), but not significantly larger than condition 17 (see Figure 5).

In summary, this study indicates that condition 14 produces more total digoxin (digoxin plus α-acetyl digoxin) on average than conditions 11, 12, 13 and 16, although not all averages are significantly different. Condition 19 seems to produce the best results compared with conditions 17 and 18. Interestingly, the conditions 14 and 19 are very similar (cold nights, sun screen, use of fertilizer and no milled soil), which
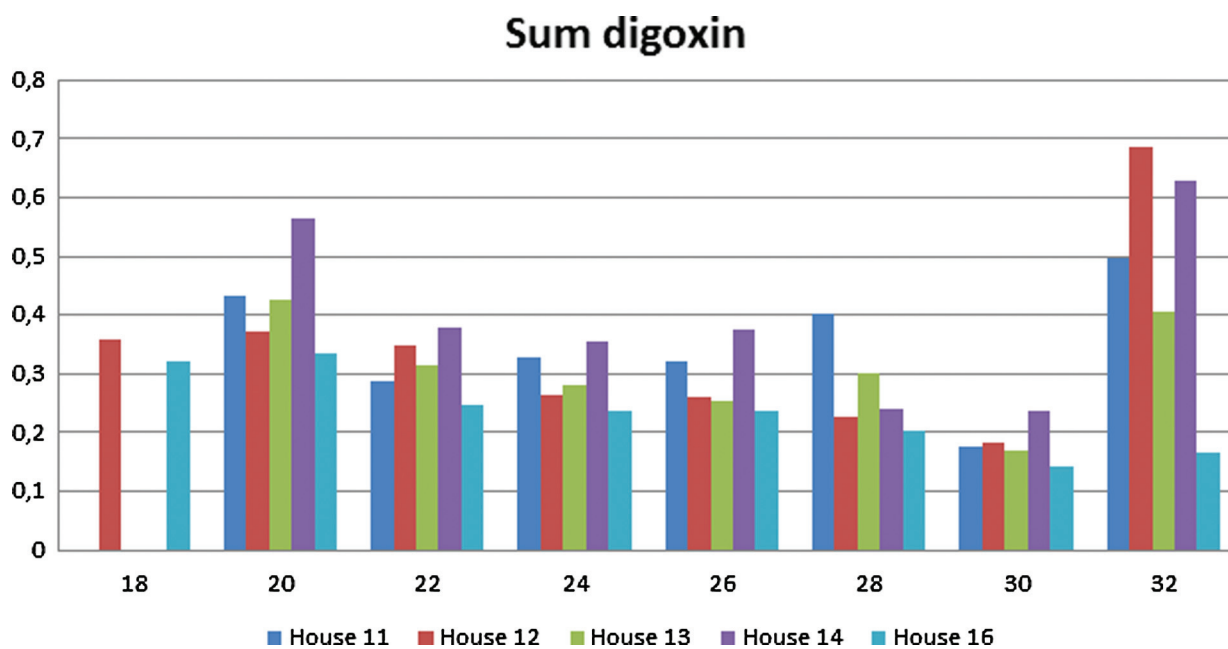
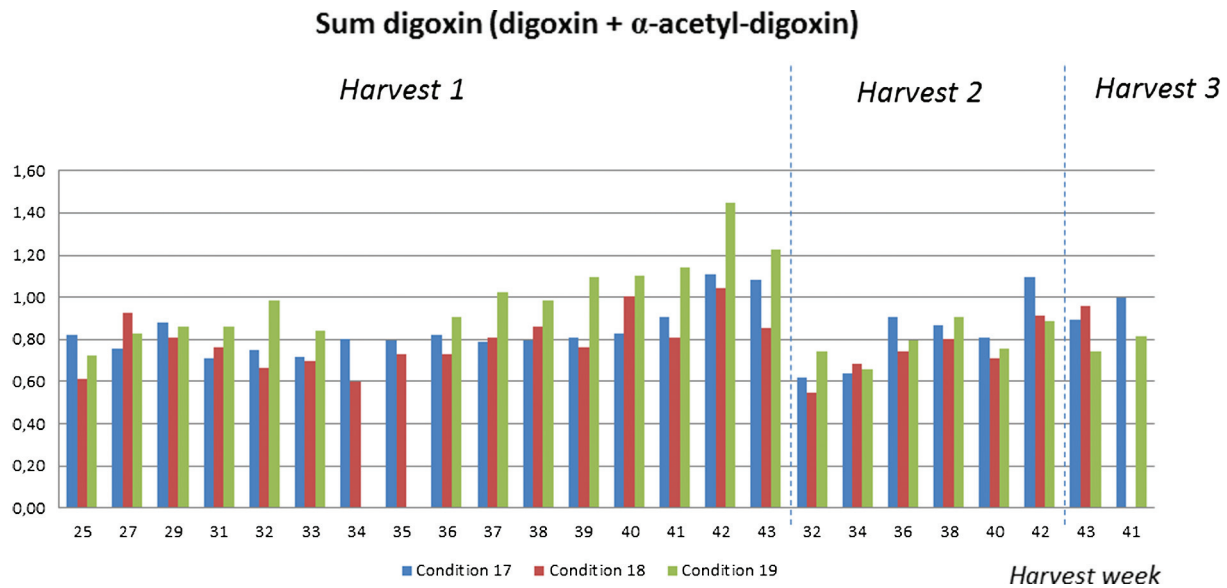**Figure 3.** Sum of digoxin and α-acetyl-digoxin for each harvest time point, for conditions 11, 12, 13, 14 and 16.

Sum digoxin (digoxin + α-acetyl-digoxin)

Figure 4. Sum of digoxin and α-acetyl-digoxin for each harvest time point, for conditions 17, 18 and 19.

shows that the findings are consistent. Furthermore, these results support the results of experiment 1 where the cold stressor produced the best results as well.

CONCLUSIONS

Normal soil, no CO_2 enrichment combined with a cold shock was found to be the optimal condition for producing digoxin in experiment 1. Gitoxin content was significantly lower in plants grown in this condition. Furthermore, the stressor caused by dragging a cloth over the plants (the 'Tuch' condition) was found to have no significant effect on the amount of digoxin. Also the time of harvesting showed no statistically significant differences in the production of cardenolides. In experiment 2 the optimal condition was found to be a combination of cold nights, sun screen, fertilizer use and no milled soil. The effect of cold was important in both experiments for improving digoxin production.

Overall, this study shows that digoxin production can be increased and at the same time the production of undesired cardenolides can be reduced by controlling the growth conditions of *D. lanata* Ehrh. in a greenhouse setting. This study also indicates that Chinese herbal medicines could be grown under more controlled conditions in greenhouses. Further studies will be needed to discover the optimal combination of growth conditions and determine the cost-effectiveness of applying those conditions for large scale production of Chinese medicines such as Mao Hua Yang Di Huang.

AUTHOR CONTRIBUTIONS

RP, JH, HH and MW conceived the experiments and designed the study. RP and JH carried out the experiment. HA analysed the data. HH setup the HPLC method and performed the HPLC measurements of the samples. HA,
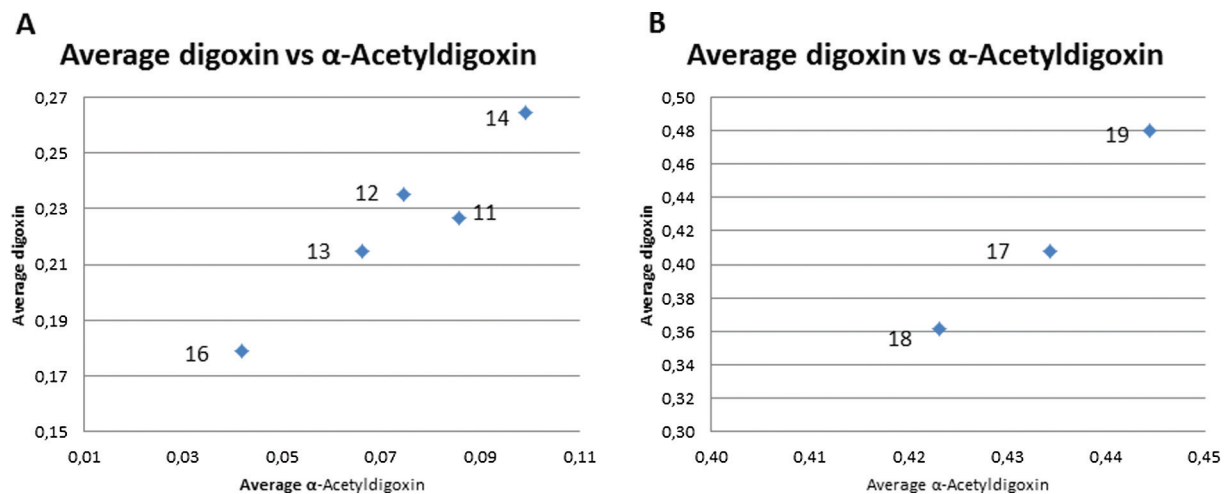

Figure 5. Average digoxin levels are plotted against average α-acetyl-digoxin levels. Panel A shows the conditions 11, 12, 13, 14 and 16. Panel B shows the conditions 18, 17 and 19.

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