Cannabinoids and zebrafish

Akhtar, M.T.

CHAPTER 4

Hydroxylation and glycosylation of Δ^9-THC by *Catharanthus roseus* cell suspension culture analyzed by HPLC-PDA and mass spectrometry.

Muhammad Tayyab Akhtar, Natali Rianika Mustafa, Robert Verpoorte, and Young Hae Choi.

Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands.

Abstract

The aim of this study was to test the capability of *Catharanthus roseus* suspension cultured cells for converting Δ^9-THC into more polar derivatives. The transformed metabolites were analysed and isolated by HPLC. Structures of some new derivatives were proposed on the basis of molecular ion peaks, fragmentation pattern and spectroscopic data obtained from LC-MS. Δ^9-THC was rapidly absorbed by *Catharanthus roseus* cultured cells and upon biotransformation new glycosylated and hydroxylated derivatives were isolated by preparative HPLC and identified using LC-MS molecular ion peaks, fragmentation patterns and spectroscopic data. In addition, cannabinol was detected as degradation product, including its glycosylated derivative. In the compound uptake efficiency, Δ^9-THC was rapidly absorbed by *Catharanthus roseus* cell suspension cultures line CRPP. Based on these results, it is concluded that *Catharanthus* cultured cells have great potential to transform Δ^9-THC into more polar derivatives and can be used for the large scale production of new cannabinoids, which can be a source of new compounds with interesting pharmacological profiles.

Introduction

Besides microbial and fungal cultures, plant cell cultures have also been utilized for biotransformation. Even though plant cell cultures most often have less capacities and resources to produce specific secondary metabolites compared to intact plants, they often retain the ability to perform all biocatalytic steps and are well able to efficiently transform exogenously applied substrates into products of interest. Moreover, it is often noted that plant cell cultures can take up and transform xenobiotic compounds according to metabolic pathways of endogenous metabolites with structural similarities, which may well lead to novel and valuable products with improved properties regarding stability, bio-

activity and solubility (Ishihara et al. 2003). Some attempts have also been made to test the plants and plant cells for the transformation of cannabinoids into new chemical entities. For example, some suspension cultures of *Cannabis sativa* (*C sativa*) and *Saccharum officinarum* were used to convert cannabidiol to cannabeilsoin (Braemer et al. 1987, Hartsel et al. 1983) and tetrahydrocannabinol to cannabicoumaron (Braemer and Paris 1987).

Catharanthus roseus cell enzyme system has been successfully used for the development of new compounds by the hydroxylation and glycosylation of the parent compound into its derivatives with enhanced water solubility. Furuya et al. (1992) reported the epoxidation of tabersonine into lochnericine and further methoxylation into lochnerinine by using a suspension culture of *C. roseus*. *Catharanthus roseus* cell suspension culture spiked with vanillin was able to convert the compound into glucovanillin (Sommer et al. 1997, Yuana et al. 2002), vanillic acid and glucovanillic acid (Yuana et al. 2002). Feeding/elicitation of a *C. roseus* cell suspension culture with salicylic acid resulted in hydroxylation of this compound into 2.5-dihydroxybenzoic acid (gentisic acid) followed by a glycosylation of the hydroxyl group at C-5 (Shimoda et al. 2002, Mustafa et al. 2009). *Catharanthus roseus* was also used to transform triptolide (Ning et al. 2004) and cinobufagin (Ye et al. 2003) into the new products 12β, 13α-dihydroxytriptonide and 3-epi-desacetylcinobufotalin respectively. Deoxyartemisinine was obtained from a suspension culture of *C. roseus* after feeding the antimalarial compound artimisinine (Patel et al. 2010). The aim of the current study is to investigate the potential of *C. roseus* cultured cells for the transformation of Δ^9-THC for producing more polar compounds, while maintaining pharmacological activity. In addition, we studied the effect of the compound on the biomass accumulation of the cell cultures and the effect of growth conditions on the biotransformation

of THC. LC-MS with APCI was employed for analytical evaluation and characterisation of Δ^9-THC derivatives.

Material and Method

Isolation of Δ^9-THC

Δ^9-THC was purified according to Hazekamp et al. (2004).

Plant cell cultures:

Catharanthus roseus cell suspension culture line CRPP was grown in Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 2% glucose and 1.86 mg/L naphthalene-3-acetic acid (NAA). The cells were grown in 250 mL-Erlenmeyer flasks containing 100 mL of the medium and cultivated at 24 (± 1)°C under continuous light (500-1500 lux) at 100 rpm. Subculturing the cells for maintenance was performed every 3 weeks by diluting the cultures 1:1 with fresh medium. For the experiment, 30 ml of the 3-week old cultures were pipetted into 50 ml fresh medium in a 250 mL-Erlenmeyer flask.

Feeding experiments:

For the feeding experiment 18 cultures were inoculated as described above and three medium flasks were maintained at similar conditions; treatments were performed in triplicate for all conditions at day five after subculturing, which corresponded to mid-exponential phase. The cell cultures received either Δ^9-THC (8 mg; dissolved in 0.8ml of ethanol at 24 and 48h), ethanol as solvent control (0.8ml) for 24 and 48h or remained untreated as non-fed control for 24 and 48h. Subsequently cells and medium were harvested at 24 and 48 hours after treatment. The medium flasks were spiked with Δ^9-THC at the same time and collected after 48 hours to determine effect of medium, pH and light on Δ^9-THC Cells were harvested by vacuum filtration using a Bűchner funnel and Whatman filter paper (d=90 mm; Ref No: 10311809, Whatman GmbH, Dassel,

Germany) ; after_initial filtration the medium was collected and cells were washed with 100 ml of Millipore water. The cells were transferred into 50 ml falcon tubes, frozen in liquid nitrogen and stored at -20 °C before further analysis.

Extraction

Collected cells were lyophilized and weighed. Fifty mg of cells (DW) were weighed into a 1,5 mL microtube and extracted with 1 ml methanol by vortexing (1 min) followed by ultrasonication for 15 minutes (at room temperature). The supernatants were collected by centrifugation (20 min at 13,000 rpm), and extraction of the remaining cell biomass was repeated twice, each time using 1 ml methanol. The pooled supernatant was evaporated to dryness and dissolved in 1 ml of ethanol p.a for LC-MS analysis. Media were extracted three times with an equal volume of EtOAc (50 ml). The media extracts were dried with a rotary evaporator, weighed, and dissolved in 1 ml ethanol p.a.; 10 ul was injected for HPLC analysis.

Hydrolysis of extracts

Cell extracts were hydrolysed in order to release Δ^9-THC from its glucoside forms and for confirmation of the glucosylated products. For this purpose, 1 mg cells (DW) from the Δ^9-THC -fed cultures were weighed into 1,5 ml microtubes and extracted with 0.5 ml methanol combined with 0.5 ml of 6 N HCl. The mixture was placed in a water bath at 80°C for 1 h (Meuwly and Metraux 1993). The hydrolysed samples were extracted with 1 ml of chloroform (three times), the pooled chloroform fractions were dried under a nitrogen flow, re-dissolved in ethanol and 10 µl injected to the LC-MS system.

HPLC Conditions

All quantitative analysis were carried out on an Agilent 1200 HPLC system equipped

with an autosampler, injector and a photo diode array (PDA) detector. A Phenomenex C18 (150 x 4.6 mm, 5µm particle size) column was used for chromatographic analysis. The mobile phase consisted of methanol and water, acidified with 25 mM of formic acid. The column was run with a gradient of methanol (65 to 100%) over 25 minutes. The column was further run for 3 min with 100% methanol followed by re-equilibration of the column under initial conditions for 4 min. Flow rate was 1.0 ml/min and the UV detection was set at 228 nm.

Isolation of some target compounds were performed using a preparative Phenomenex C18 (250 x 4.6 mm, 5 µm particle size) column using an isocratic elution system with MetOH-water (85-15) with a run-time of 25 minutes, a flow-rate of 2.0 ml/min and detection at 228 nm.

LC-MS Analyses

LC-MS analyses for product identification were performed by using an Agilent 1100 HPLC single-quadrupole mass-spectrometer. Instrument operation and data acquisition were controlled through Agilent LC/MSD Chemstation A.10.02 software (Santa Clara,CL, United states). The HPLC system and column conditions were the same as mentioned above. The settings of the mass spectrometer (MS) were as follows: APCI mode; Positive and negative ionization; fragmenter voltage, 150 and 250 V; gas temperature, 350C; vaporizer temperature, 350 $^{\circ}$C; drying gas (N_2) flow rate, 5 liters min^{-1}; nebulizer gas pressure, 35 psig (lb/in^2); capillary voltage, 4000 V; corona current, 4.0 µA. Ions were detected in the range of 100-1000 u.

Statistical analysis

Statistical analyses were performed using GraphPad Prism for Windows (version 5.03) and also used to plot graphs. To analyze the impact of EtOH and

Δ^9-THC on total dry cell weight, we used one-way analysis of variance and a Dunnett's Multiple comparison test with probability level of 5% as the minimal criterion of significance.

Results and discussion

Quantitative analysis of Δ^9-THC in the media and the cells was performed in order to study the efficiency of uptake of this compound by the *C. roseus* cells line CRPP. The presence of a small proportion of the compound/substrate in the cells and only traces in the media (Figure 1A) shows the quick uptake of compound by the *C. roseus* cells. Further, the transformed products were released back into the media. The effect of growth conditions (temperature, light, humidity etc.) on the stability of Δ^9-THC was studied by incubating the media spiked with Δ^9-THC for 48 h (as negative control). The identification and quantification of cannabinol (CBN) from these flasks showed that 28 to 30 % of the Δ^9-THC was degraded to CBN only because of the growth conditions.

The effect of Δ^9-THC on the growth of *Catharanthus* cell line was studied by measuring the fresh weight and dry weight of the cells fed with Δ^9-THC for 24 and 48 h, and compared with those of the control cells which were treated with ethanol only (as control solvent) or not treated at all (control blank). The dry weight of the cells treated with Δ^9-THC was significantly reduced as compared to the controls, which could be due to toxicity of Δ^9-THC to the cells. A longer treatment with Δ^9-THC (48h) further reduced the dry weight of the cells slightly (Figure 1B).

Transformation of product

The potential of *Catharanthus roseus* cell suspension culture as tool for drug transformation is well documented (Bourgogne et al. 1989). For example, the

transformation of cinobufagin was performed using cell suspension culture of *C. roseus* and its four glycosylated derivatives were identified (Ye et al. 2002).

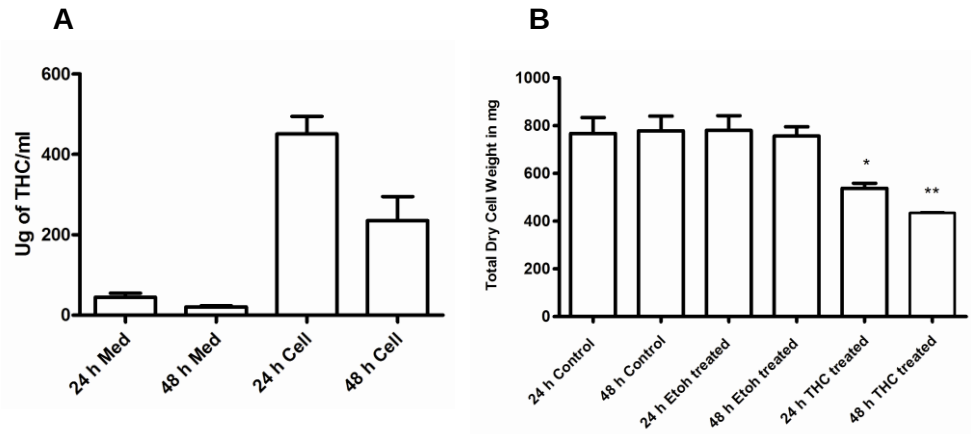

Figure 1: A, Quantification of Δ^9-THC (ug/ml) in media and cells after 24 and 48 hours of incubation. **B**, Total dry cell weight of CRPP incubated in 11-Glu media (Control), treated with EtOH and Δ^9-THC for 24 and 48 hours. ***** depict differences between 24h control and cells treated with EtOH and Δ^9-THC for 24 and 48 hours. Statistical icons: *= p< 0.05, **= p< 0.01.

Previously, cannabinoids were transformed by using different plant cell cultures; however, there is no report on the use of *C. roseus* as a transformation tool for cannabinoids. The Δ^9-Tetrahydrocannabinol (**1)** was transformed into four different derivatives named as **2, 3, 4** and **5** (Figure 2). Structures are proposed on the basis of molecular mass, fragmentation pattern, and spectroscopic data of the transformed compounds obtained from LC-MS.

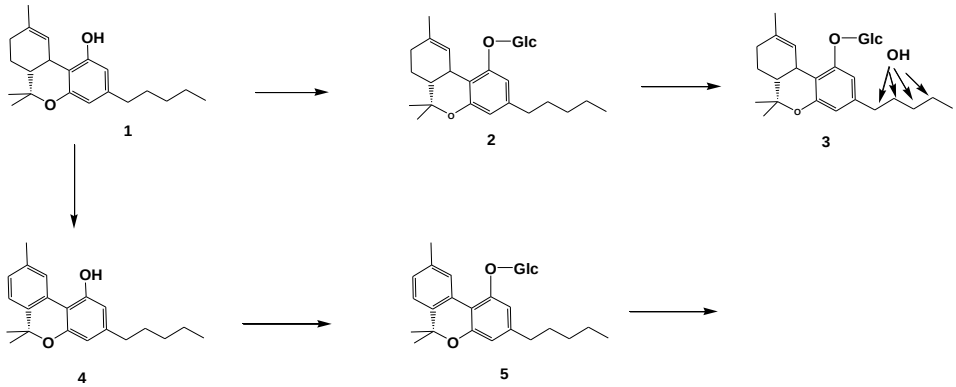

Figure 2: Chemical structures of products 1, 2, 3, 4 and 5.

The compound **1** was transformed to its glycosylated and further hydroxylated derivative **2** and **3** respectively. The positive parent ion peak at 477 $[M+H]^+$ (Figure 3A) is indicating the addition of one glucose molecule to compound **1** and its fragmentation to base peak at 315 $[M+H]^{+,}$ suggested that **2** is composed of **1** and one molecule of glucose. The compound **3** eluted before **2** with a molecular ion peak at 493 $[M+H]^+$ (Fig. 3B) proposing a further addition of an hydroxyl group to compound **2**. This was also confirmed by the fragmentation of this derivative in which **3** was fragmented to m/z 331 and 313. The positive peak at m/z 331 indicates the removal of a glucose molecule resulting in a molecular weight which is a sum of compound **1** and a hydroxyl group. After the hydrolysis of the EtOAc extract (medium extract), molecular peaks of m/z 331 and m/z 315 were found. These peaks are evidence that after loss of glucose molecule, either **1** only or its hydroxyl derivative was present in the extract. The hydroxyl group is probably attached to the 5 carbon side chain or non-phenolic ring of the product **3**. Literature shows that the chromophore of the cannabinoids corresponds to its substituted phenolic ring, whereas alkyl-side chain and cyclization of non-phenolic part has no influence on the absorbance (Hazekamp et al. 2005). In the current study, spectroscopic data show the same

UV-spectrum (Figure 4, 5) but different retention times for the products **1**, **2** and **3**. The elution sequence was **3**, **2** and **1**.

An earlier study had revealed that **1** is quite an unstable compound at high temperature, light and acidic medium (Tanaka et al. 1993). This was confirmed by chromatographic and spectroscopic analyses which showed that after an exposure of 48 h to light and a temperature of 35 °C in the presence of 11-Glu media, 28-30 % of **1** was degraded into 4. The product 4 was quantified by making the calibration curve of the reference compound and obtained the r^2-value equal to 0.998. The positive molecular ion peak at m/z 473 $[M+H]^+$ (Fig. 3D) shows that 4 was further converted to product 5 with an addition of a

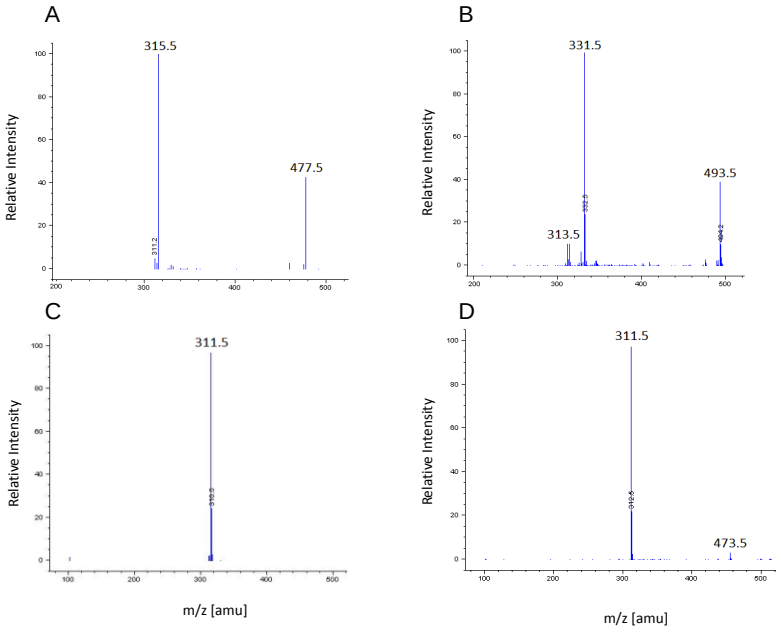

Figure 3: LC-MS spectra of the isolated products. A) Product 2; B): Product 3; C): Product 4; D): Product 5.

glucose molecule. The MS fragmentation of **5** also confirms that m/z 473 $[M+H]^+$ was fragmented to a base peak at m/z 311 $[M+H]^+$, which is the molecular weight of compound **4**. This assumption was also verified by the

acidic hydrolyses of 5 which released a glucose molecule and a molecular ion peak at m/z 311 was found. The UV spectrum of compound **4** (Fig. 4C) and **5** (Fig. 4D) is similar to that of CBN reference compound (Fig. 5B). Previously, the tissue cultures of tubers of *Pinelli ternate* were reported to be able to transform Δ^8-tetrahydrocannabinol and cannabinol to their glycosylated derivatives (Tanaka et al. 1993, 1997). Our data is in good agreement with these previous reports and shows that the CRPP cell line has not only transformed **1** but interestingly, its enzymatic system can also efficiently convert **4** to its glycosylated derivative. Although, **1** was degraded to **4** because of growth but interestingly, its enzymatic system can also efficiently convert **4** to its glycosylated derivative. Although, **1** was degraded to **4** because of growth

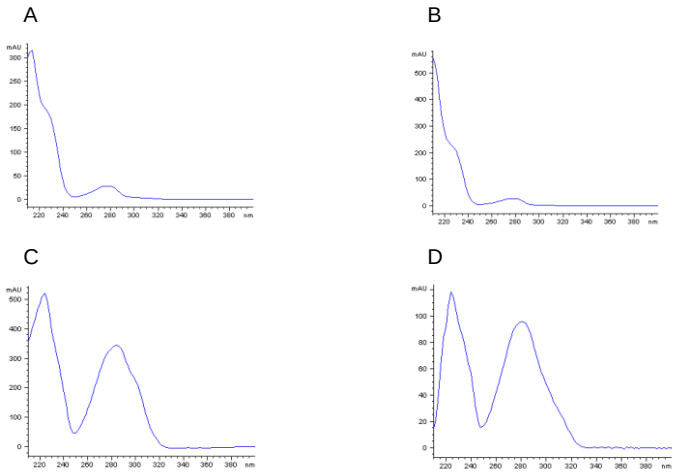

Figure 4: UV-spectra of the isolated products in the range of 190-400 nm. A) Product 2; B): Product 3; C): Product 4; D): Product 5.

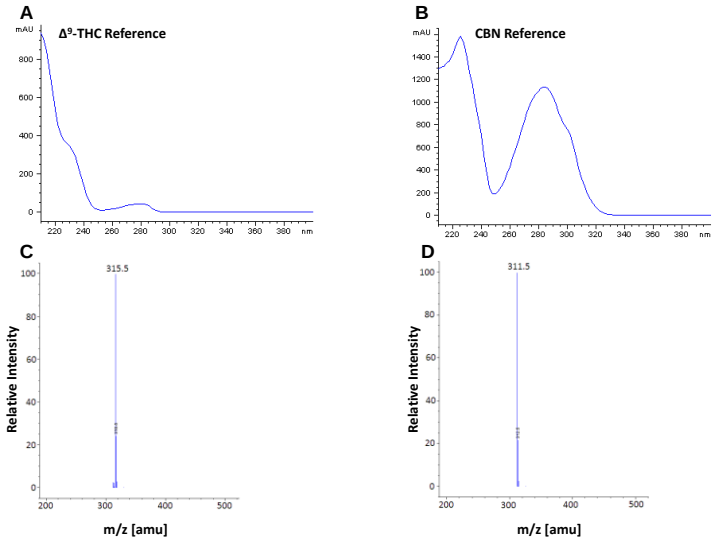

Figure 5: LC-MS and UV spectra of Δ^9-THC and CBN reference compounds. A): Δ^9-THC UV spectra; B): CBN UV spectra; C): Δ^9-THC MS spectra; D): CBN MS spectra.

conditions but its further glycosylation was done in the presence of CRPP cultured cells. Compound **3** was the major compound indentified in the EtOAc extract, whereas 4 and 5 were minor compounds. The low quantity of **4** and **5** might be due to the fast uptake of **1** leaving only very little amounts to be degraded into **4**. *Pinelli ternata* tissue segments also successfully transformed cannabidiol and cannabidiolic acid into their glycosylated derivatives. Cannabidiol was transformed into cannabidiol- *O-β*-D-glucopyranoside and cannabidiol- *O-β*-D-diglucopyranoside. In both derivatives, glucose molecules were conjugated with the phenolic hydroxyl group (Tanaka et al. 1996). These studies suggest that the phenolic hydroxyl group is the most favorable site for glucose linkage in the transformation of cannabinoids.

Conclusion

The rapid uptake and conversion of Δ^9-THC into glycosylated and further hydroxyl derivatives shows the conversion capabilities of *Catharanthus roseus* cell suspension culture line CRPP. A growing body of literature including data presented in this study establishes that the glycosylation of cannabinoids is a common metabolic pathway in plant tissue or cell cultures. Glycosylated cannabinoids are phase ii reaction products in mammals and could easily be produced on larger scale by using cell suspension cultures of *C. roseus*. However, we incubated cells for two days after adding the substrate. Larger quantity of substrate and longer incubation time in dark conditions could minimize the substrate degradation and might be helpful to obtain a higher quantity of new derivatives for NMR studies.

References

Abbott BJ, Fukuda DS, Archer RA. 1977. Microbiological transformation of cannabinoids. Experientia 33: 718-720.

Binder M, Meisenberg G. 1978. Microbial transformation of cannabinoids 2. A screening of different microorganisms. Eur J Appl Microbiol Biotechnol 5: 37-50.

Bourgogne V, Labidalle S, Galons H, Miocque M, Foulquier M, Jacquin-Dubreuil A, Cosson L. 1989. Biotransformation of a synthetic compound, 1,5-diphenylsulphinyl-3-methyl-3-nitropentane, by cell suspensions of *Catharanthus roseus*. Phytochemistry 28: 2345-2347.

Braemer R, Paris M. 1987. Biotransformation of cannabinoids by a cell suspension culture of *Cannabis sativa* L. Plant Cell Rep 6: 150-152.

Breithaupt D, Kraut S. 2006. Simultaneous determination of the vitamins A, E, their esters and coenzyme Q_{10} in multivitamin dietary supplements using an RP-C30 phase. Eur Food Res Technol 222: 643-9.

Carter GT, Weydt P. 2002. Cannabis: Old medicine with new promise for neurological disorders. Curr Opin Investig Drugs 3: 437-440.

Chen G, Pramanik BN, Liu YH, Mirza UA. 2007. Applications of LC/MS in structure identifications of small molecules and proteins in drug discovery. J Mass Spectrom 42: 279-87.

Fitzgerald RL, Rivera JD, Herold DA. 1999. Broad spectrum drug identification directly from urine, using liquid chromatography-tandem mass spectrometry. Clin Chem 45: 1224-34.

Fukuda D, Archer RA, Abbott BJ. 1977. Microbiological transformations of {Delta}6a,10a-tetrahydrocannabinol. Appl Environ Microbiol 33: 1134-1140.

Furuya T, Sakamoto K, Iida K, Asada Y, Yoshikawa T, Sakai S, Aimi N. 1992. Biotransformation of tabersonine in cell suspension cultures of *Catharanthus roseus*. Phytochemistry 31: 3065-8.

Gamborg OL, Miller RA, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158.

Gaoni Y, Mechoulam R. 1966. Cannabichromene, a new active principle in hashish. Chem Commun 1: 20.

Hartsel SC, Loh WHT, Robertson LW. 1983. Biotransformation of cannabidiol to cannabielsoin by suspension-cultures of *cannabis-sativa* and *Saccharum-officinarum*. Planta Med 48: 17-19.

Hazekamp A, Peltenburg A, Verpoorte R, Giroud C. 2005. Chromatographic and Spectroscopic Data of Cannabinoids from *Cannabis sativa* L. J Liq Chromatogr Relat Technol 28: 2361-2382.

Hazekamp A, Simons R, Peltenburg-Looman A, Sengers M, van Zweden R, Verpoorte R. 2004. Preparative Isolation of Cannabinoids from *Cannabis sativa* by Centrifugal Partition Chromatography. J Liq Chromatogr Relat Technol 27: 2421-2439.

Meuwly P, Metraux JP. 1993. Ortho-anisic acid as internal standard for the simultaneous quantitation of salicylic acid and its putative biosynthetic precursors in cucumber leaves. Anal Biochem 214: 500-5.

Michael B, Astrid P. 1980. Microbial transformation of cannabinoids. Part 3: Major metabolites of (3R,4R) -Δ^1-tetrahydrocannabinol. Helv Chima Acta 63: 2515-2518.

Mustafa NR, Kim HK, Choi YH, Verpoorte R. 2009. Metabolic changes of salicylic acid-elicited *Catharanthus roseus* cell suspension cultures monitored by NMR-based metabolomics. Biotechnol Lett 31: 1967-1974.

Ning LL, Han J, Zhang XY, Guo HZ, Bi KS, Guo DA. 2004. Biotransformation of triptolide and triptonide by cell suspension cultures of *Catharanthus roseus*. J Asian Nat Prod Res 6: 93-7.

Patel S, Gaur R, Verma P, Bhakuni RS, Mathur A. 2010. Biotransformation of artemisinin using cell suspension cultures of *Catharanthus roseus* (L.) G.Don and Lavandula officinalis L. Biotechnol Lett 32: 1167-71.

Pitt JJ. 2009. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. Clin Biochem Rev 30: 19-34.

Rashidi H, Akhtar MT, Kooy FDR, Verpoorte R, Duetz WA. 2009. Hydroxylation and further oxidation of Δ^9-tetrahydrocannabinol by alkane-degrading bacteria. Appl Environ Microbiol 75: 7135–7141.

Shimoda K, Yamane S-y, Hirakawa H, Ohta S, Hirata T. 2002. Biotransformation of phenolic compounds by the cultured cells of *Catharanthus roseus*. J Mol Catal B: Enzym 16: 275-281.

Sirikantaramas S, Taura F, Morimoto S, Shoyama Y. 2007. Recent Advances in *Cannabis sativa* Research: Biosynthetic Studies and Its Potential in Biotechnology. Curr. Pharm. Biotechnol 8: 237-243.

Sommer J, Schröder C, Stöckigt J. 1997. *In vivo* formation of vanillin glucoside. Plant Cell Tiss Org Cult 50: 119-123.

Tanaka H, Morimoto S, Shoyama Y. 1993. Cannabis, 21. Biotransformation of Cannabinol to Its Glycosides by In Vitro Plant Tissue. J Nat Prod 56: 2068-2072.

Tanaka H, Takahashi R, Morimoto S, Shoyama Y. 1996. Cannabis 25, biotransformation of cannabidiol and cannabidiolic acid by *Pinellia ternata* tissue segments. Plant Cell Rep 15: 819-823.

Tanaka H, Takahashi R, Morimoto S, Shoyama Y. 1997. A new Cannabinoid, Δ^6-Tetrahydrocannabinol 2'-*O-beta*-D-glucopyranoside, Biotransformed by Plant Tissue. J Nat Prod 60: 168-170.

Xiao JF, Zhou B, Ressom HW. 2012. Metabolite identification and quantitation in LC-MS/MS-based metabolomics. Trends Analyt Chem 32: 1-14.

Ye M, Dai J, Guo H, Cui Y, Guo D. 2002. Glucosylation of cinobufagin by cultured suspension cells of *Catharanthus roseus*. Tetrahedron Lett 43: 8535-8538.

Ye M, Ning L, Zhan J, Guo H, Guo D. 2003. Biotransformation of cinobufagin by cell suspension cultures of *Catharanthus roseus* and *Platycodon grandiflorum*. J Mol Catal B-Enzym 22: 89-95.

Yuana, Dignum MJW, Verpoorte R. 2002. Glucosylation of exogenous vanillin by plant cell cultures. Plant Cell Tiss Org Cult 69: 177-182.

Ishihara K, Hamada H, Hirata T, Nakajima N. 2003. Biotransformation using plant cultured cells. J Mol Catal B-Enzym 23: 145-70.