Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 303–312
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Liquid chromatography–mass spectrometry studies of St. John's wort methanol extraction: active constituents and their transformation☆

Frances Liu[a], Changkang Pan[a,*], Patrick Drumm[a], Catharina Y.W. Ang[b]

[a] *Novartis Pharmaceuticals Corporation, East Hanover, NJ 07936, USA*
[b] *National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR 72079, USA*

Received 2 August 2004; received in revised form 21 October 2004; accepted 28 October 2004
Available online 9 December 2004

Abstract

The influence of light and solution pH on the stability behavior of phloroglucinols (hyperforin and adhyperforin) and naphthodianthrones (hypericin, pseudohypericin, protohypericin and protopseudohypericin) extracted with methanol from St. John's wort powder (*Hypericum perforatum* L.) were studied using liquid chromatography–mass spectrometry (LC–MS). When exposed to light, hyperforin and adhyperforin in this extract solution degraded rapidly, particularly at pH 7, where within 12 h complete transformation was observed. Contrastingly, when protected from light, the solutions regardless of pH, underwent minimal transformation after 36 h. Under light and neutral pH conditions, phloroglucinols and naphthodianthrones had different stability behaviors, which were attributed to the different oxidation mechanisms. Four experiments performed on naphthodianthrones exhibited serious transformation at acidic pHs. One hyperforin transformation product was studied using LC–MS. The molecular structure was proposed on the basis of ion fragmentation patterns obtained from MS/MS studies.

Keywords: Hypericum perforatum L.; St. John's wort; Hyperforins; Hypericins; LC/MS identification

1. Introduction

Over the past two decades, St. John's wort (*Hypericum perforatum* L.) has been the subject of research aimed at establishing the chemical and pharmacological basis of the antidepressant activity of its extracts. St. John's wort contains groups of naphthodianthrones and phloroglucinols, which contribute to its antidepressant activity. Numerous papers have been published in the areas of isolation and purification of hyperforin [1], supercritical carbon dioxide extraction [2], HPLC analytical assay [3–5], capillary electrophoresis [6], optimization of extraction conditions [7], NIR rapid quantification [8], characterization of hyperforin transformation products using mass spectrometry [9–14] and nuclear magnetic resonance (NMR) spectroscopy [15–19]. ^{1}H NMR and ^{13}C NMR spectra are often the preferred techniques for elucidation of transformation products due to their detailed structural information. Wolfender et al. recently conducted structural elucidation of isomeric oxidized forms of hyperforin using both LC–MS and LC–NMR [19]. Typically, NMR analysis requires a labor-intensive separation and isolation process from a relatively large quantity of materials. Additionally, at times it may not be clear whether the isolated compound is the original transformation product or an artifact resulting from the extraction or isolation processes. LC–MS can be a powerful tool for structure elucidation of transformation products due to its high sensitivity, fast and direct analysis capability, and structure-rich fragmentation pattern. The technique has been widely used in the pharmaceutical industry to identify drug degradation products, impurities, and by-products. The use of LC–MS for identification of hyperforin transformation products provides a fast and direct analysis, which is the major benefit of LC–MS compared to NMR. Although a number of papers were published on the application of LC–MS to the St. John's wort extraction [9–14], most of the studies focused on the quantitative applications.

☆ The views presented in this article do not necessarily reflect those of the US Food and Drug Administration.

* Corresponding author. Tel.: +1 862 778 4956; fax: +1 973 781 2019.
E-mail address: charles.pan@pharma.novartis.com (C. Pan).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.10.034

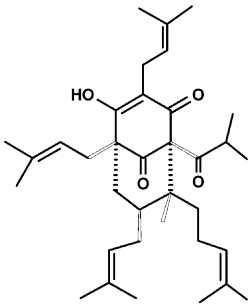
Recently, Fuzzati et al. [10] conducted LC–MS/MS studies of St. John's wort extraction and proposed several hyperforin transformation products.

The molecular structures of six active constituents in the St. John's wort are illustrated below [5]. Based on the structural characteristics, these six compounds can be divided into two different groups: phloroglucinols and naphthodianthrones. Phloroglucinol compounds are sensitive to oxidation and unstable in solutions, especially when exposed to light and air [9,10]. In comparison, naphthodianthrone compounds are generally more stable due to their aromatic conjugation [5], except that the proto forms are not stable under light.

The objectives of this study are to investigate the influence of light and solution pH on the stability behavior of phloroglucinols and naphthodianthrones in St. John's wort methanol extraction and to identify a hyperforin transformation compound using LC–MS. In this study methanol was chosen as the closest extraction solvent representative of ethanol, which is typically used in commercial St. John's wort manufacturing processes.

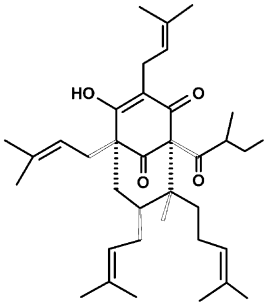
PHLOROGLUCINOLS

Hyperforin



MW : 536.3866
MF : C35H52O4

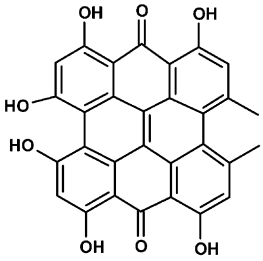
Adhyperforin



MW : 550.4022
MF : C36H54O4

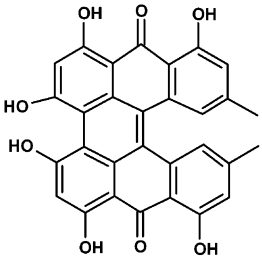
NAPHTHODIANTHRONES

Hypericin



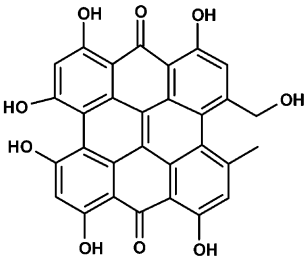
MW : 504.0845
MF : C30H16O8

Protohypericin



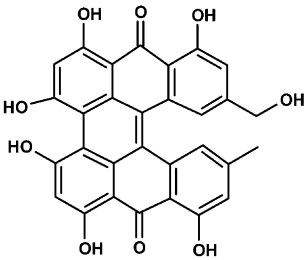
MW : 506.1002
MF : C30H18O8

Pseudohypericin



MW : 520.0794
MF : C30H16O9

Protopseudohypericin



MW : 522.0951
MF : C30H18O9

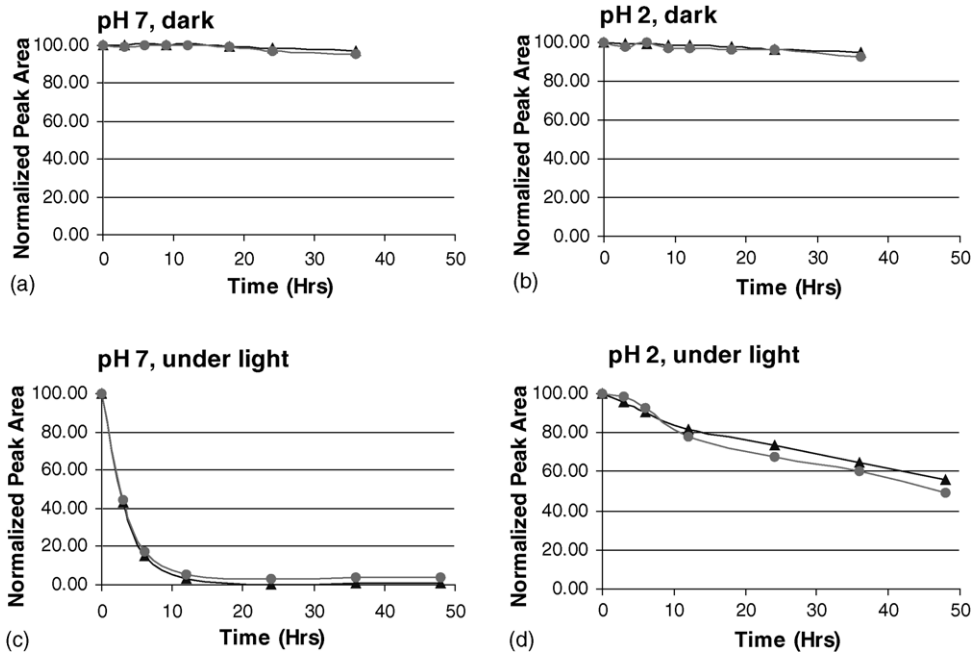


Fig. 1. Influence of light and pH on hyperforin (●) and adhyperforin (▲).

2. Experimental

2.1. Sample preparation

Methanol purchased from Fisher Scientific Co. (New Jersey, USA) was bubbled with N_2 flow for 10 min to eliminate dissolved oxygen. Dry St. John's wort powders were purchased from Frontier Natural Product Corporation (Norway, Iowa, USA). About 5 mg of dry St. John's wort powders were weighed into an Erlenmeyer flask, to which 30 mL of methanol was added, and then the flask was covered with aluminum foil to protect from room light. The powder soaked in methanol was sonicated for half-an-hour. The solution was centrifuged at 3000 rpm for 10 min to obtain clear supernatant. A 10 mL portion of the supernatant was then adjusted to form acidic solutions (pH 2) by adding the calculated amounts of hydrochloric acid 1N solution (certified, 1.005–0.995N, Aldrich Chemical Co., Milwaukee, Wisconsin, USA). The pH of un-adjusted methanol extraction was about 7 measured by pH indicator paper (Whatman Co., New

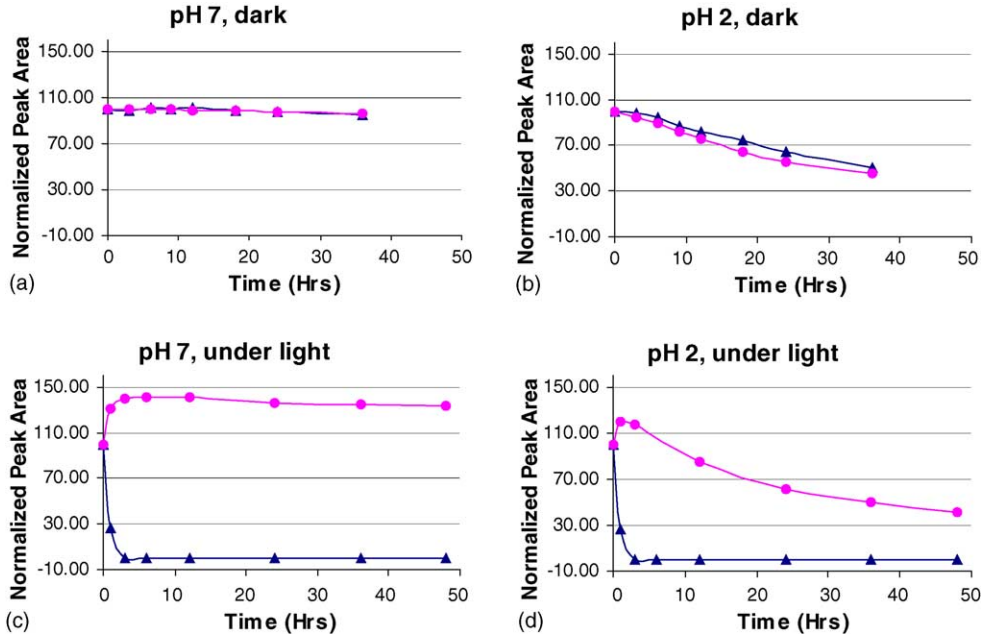


Fig. 2. Influence of light and pH on hypericin (●) and protohypericin (▲).

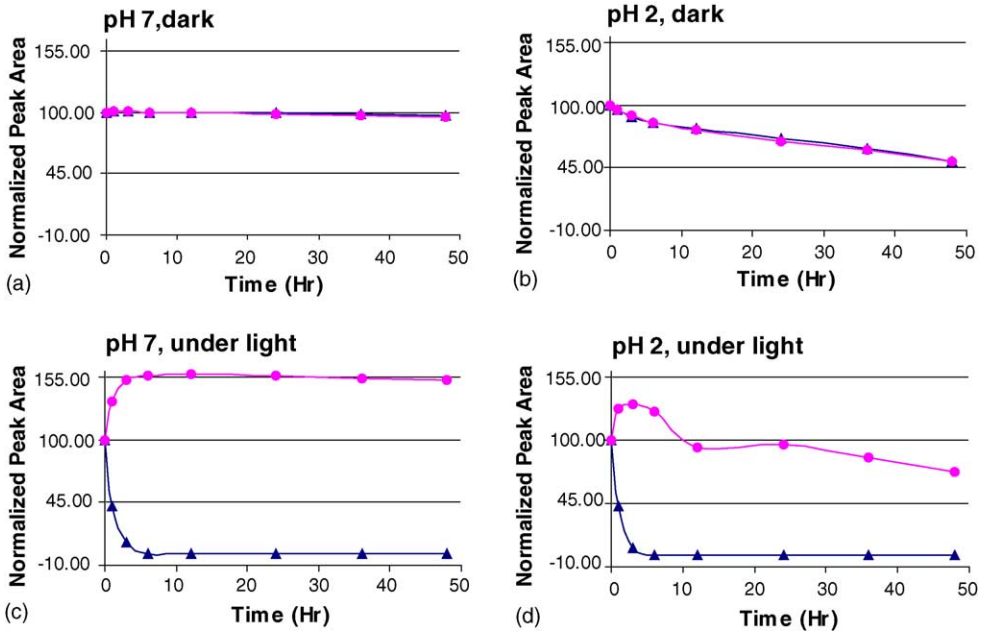


Fig. 3. Influence of light and pH on pseudohypericin (●) and protopseudohypericin (▲).

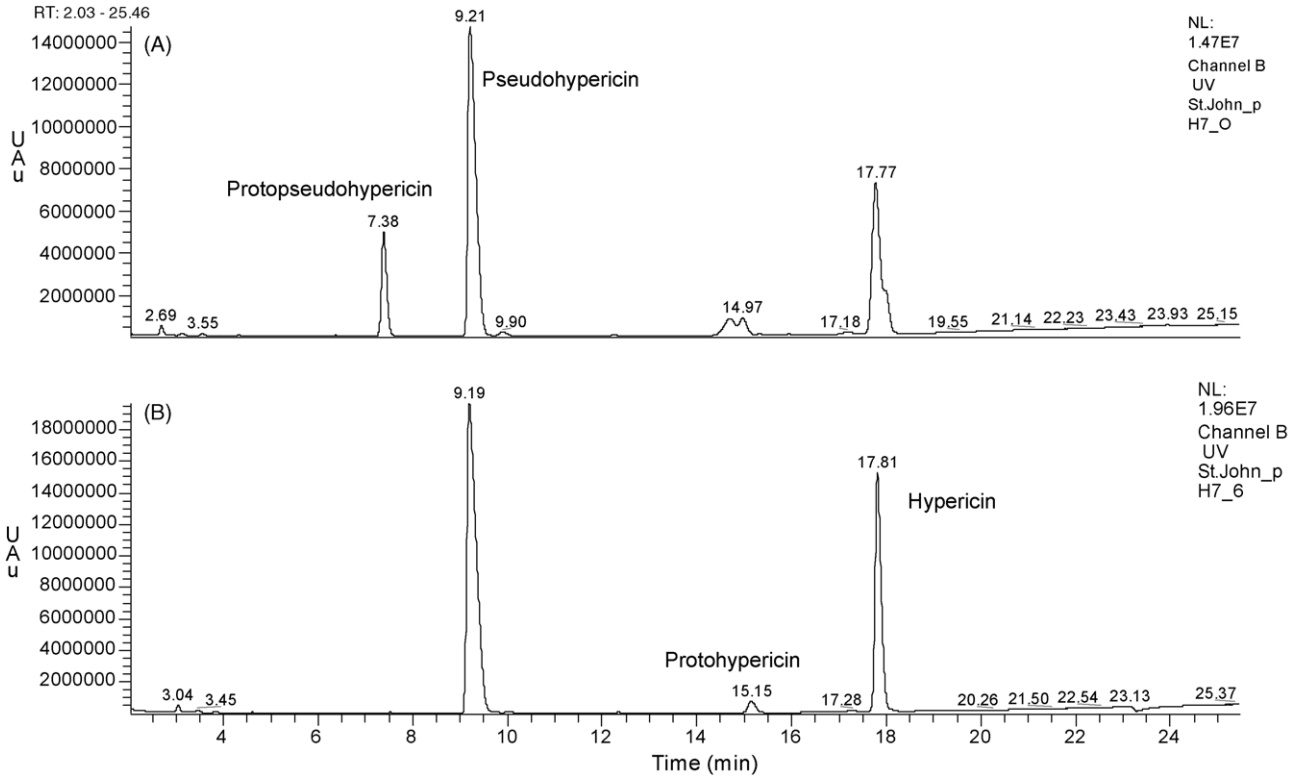


Fig. 4. (A) Chromatogram of St. John's wort methanol extraction at pH 7, initial conditions (no light exposure), detected by UV at 588 nm. (B) Chromatogram of St. John's wort methanol extraction at pH 7, light exposure for 6 h, detected by UV at 588 nm.

Jersey, USA). The extracted sample solutions were stored at room temperature (23 °C) in clear HPLC vials, which were exposed to conventional white fluorescent lamps in the laboratory. The same extraction solutions were stored in amber HPLC vials for comparison. Each of the vials was analyzed by LC–MS at set time intervals up to 48 h.

Although the standard reference substances to be studied were not available in this study, the UV spectra and MS spectra of phloroglucinols and naphthodianthrones have been well known in the literature. As a result, the identity of these compounds can be readily confirmed by LC–MS data [10,19].

2.2. HPLC

A Waters 2690 Alliance HPLC system was used in this study. The separation was achieved using a YMC-Pack Pro C_{18} 4.6 mm × 100 mm column with a particle size of 3 μm. A neutral mobile phase was used in order to run both positive and negative ionization modes in one sample injection. The mobile phases were: (A) a mixture of 20 mM ammonium acetate (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) and acetonitrile (Fisher Scientific Co., New Jersey, USA) in a ratio of 9:1; (B) acetonitrile. The flow rate was 1 mL/min with a gradient elution beginning with 50% B, changing linearly to 100% B from 2 to 22 min, and then holding 15 min before returning to the initial condition. The injection size was 15 μL and the total run time was 48 min. A diode array detector (ThermoQuest UV 6000 LP PDA) was used to monitor UV–vis signals in a spectrum ranging from 200 to 700 nm.

2.3. Mass spectrometer

All mass spectral data were collected using a LCQ^{deca} ion trap mass spectrometer (ThermoFinnigan, San Jose, California, USA) equipped with an electrospray ionization source. Both positive and negative ionization modes were operated during the acquisition to confirm the molecular weight determination. The tune conditions were set as follows: source voltage 5 kV, capillary voltage 3 V, sheath gas flow 80, auxiliary gas flow 20, capillary temperature 300 °C. The mass range from 250 to 1000 amu was acquired with a collision energy of 40 on the selected ions in the MS/MS studies.

3. Results and discussion

3.1. Stability evaluation of St. John's wort extraction

LC–MS was used to monitor the responses (peak area) of phloroglucinols and naphthodianthrones in St. John's wort

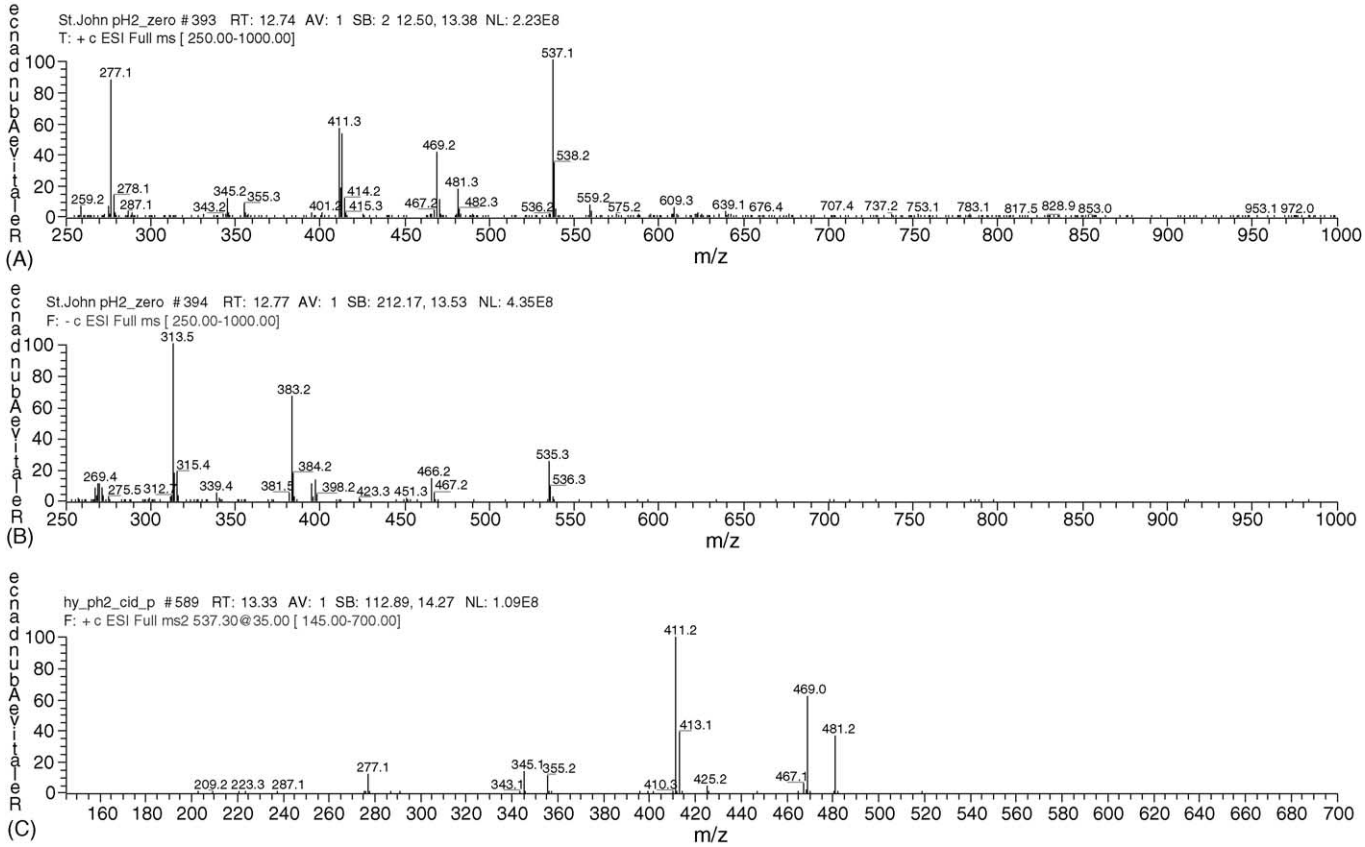


Fig. 5. (A) Positive ion mass spectrum of the peak at 12.7 min (hyperforin); (B) negative ion mass spectrum of the peak at 12.7 min (hyperforin); (C) positive ion MS/MS spectrum of *m/z* 537 (hyperforin $[M+H]^+$ ion).

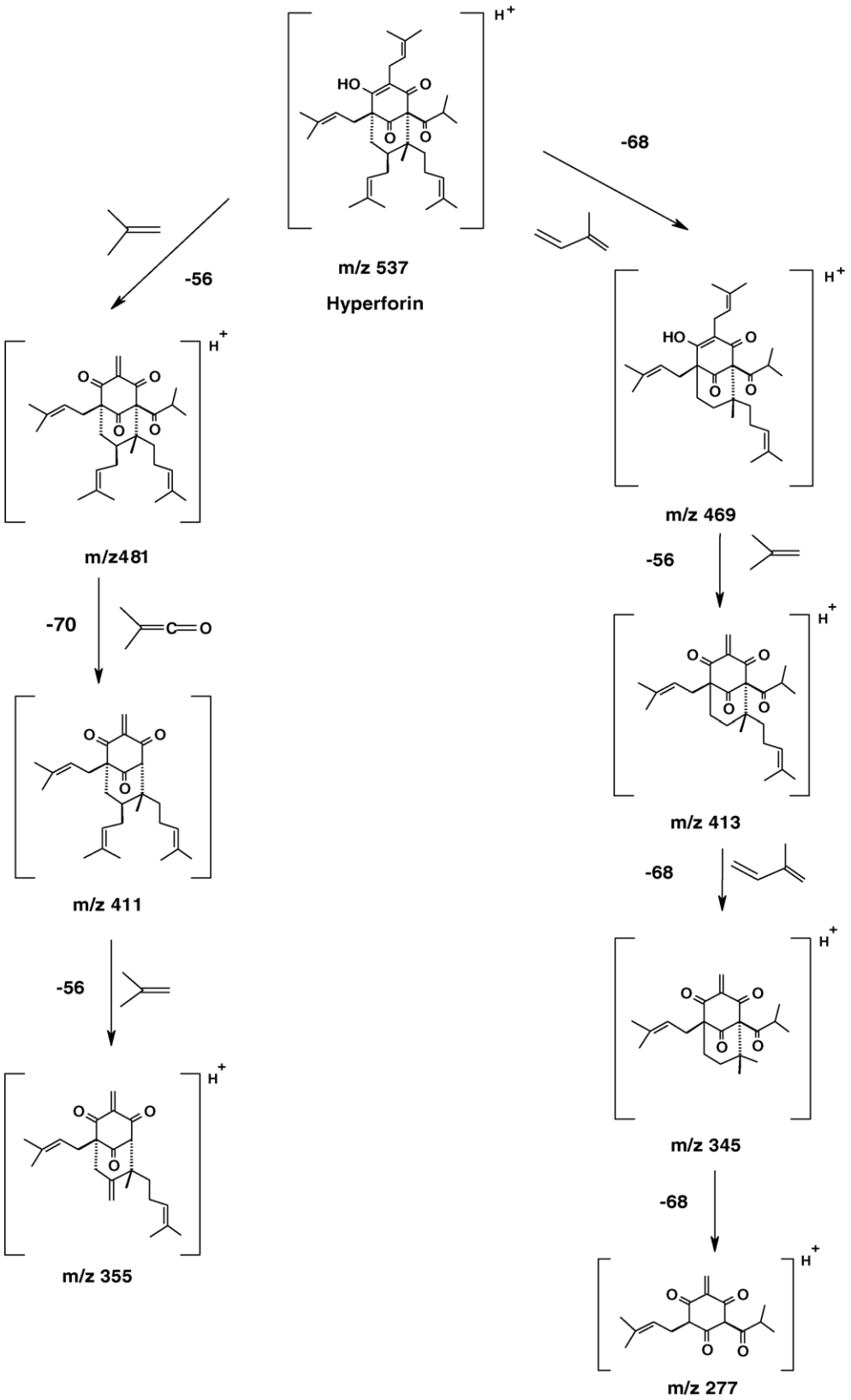


Fig. 6. Proposed fragmentation pathway of *m*/*z* 537 (hyperforin $[M + H]^+$ ion).

methanol extraction under a variety of testing conditions, such as pH and light exposure. Phlorogucinols are very weak acidic and could form both positive and negative ions. A positive ionization mode was used for phlorogucinols as it could provide more structural information. For naphthodianthrones compounds, a negative ionization mode was used as they are acidic and readily form negative ions in the electrospray ionization source.

The relative effects of light exposure and solution pH on the stability of both phloroglucinols and naphthodianthrones have been shown in Figs. 1–3. In each of these plots, the peak area responses have been normalized to their respective zero time values. As seen in Fig. 1a and b, hyperforin and adhyperforin were quite stable at pH 7 and pH 2 when protected from light. However, when the same solutions were exposed to light (Fig. 1c and d), the stability behavior became strongly pH dependent, with almost complete conversion occurring at pH 7 in just 12 h while at pH 2 approximately 50% remains after 48 h. The similar stability trends for hyperforin and adhyperforin are not surprising since their structures only differ by one methylene group.

The impact of light and solution pH on naphthodianthrone compounds can be classified into four specific cases:

In case 1, neutral pH and no light exposure (refer to Figs. 2a and 3a), neither ring formation nor transformation was observed for any of the four naphthodianthrone compounds studied. They were all quite stable, with no significant intensity change observed over 3 and 4 days. In case 2, acidic pH and no light exposure (refer to Figs. 2b and 3b), no ring formation was observed, however, decomposition did occur. These four compounds gradually degraded over time with similar decomposition rates. In case 3, neutral pH with light exposure (refer to Figs. 2c and 3c), ring formation did occur without significant other compound transformation. The two compounds with closed-ring structures (hypericin and pseudohypericin) rapidly increased at the initial period, reaching a plateau within 6 h, and then holding constant over 48 h. In comparison, protohypericin and protopseudohypericin (open-ring structures) underwent rapid and complete photocoversion with no detectable amounts present after just 6 h. In case 4, acidic pH with light exposure (refer to Figs. 2d and 3d), the most dramatic changes occurred with both ring formation and significant transformation evident. Hypericin and pseudohypericin (close-ring structures) increased quickly during the first 3 h, and then decreased continuously without forming a plateau. The presence of acid appears to cause hypericin and pseudohypericin to degrade continuously over time, whereas with protohypericin and protopseudohypericin (open-ring system) complete changes were observed in just 3 h.

Fig. 4 illustrates the chromatographic comparison of two extraction solutions measured at 588 nm, the optimal wavelength for naphthodianthrone compounds. Chromatogram A is the initial solution at pH 7 without light exposure. All

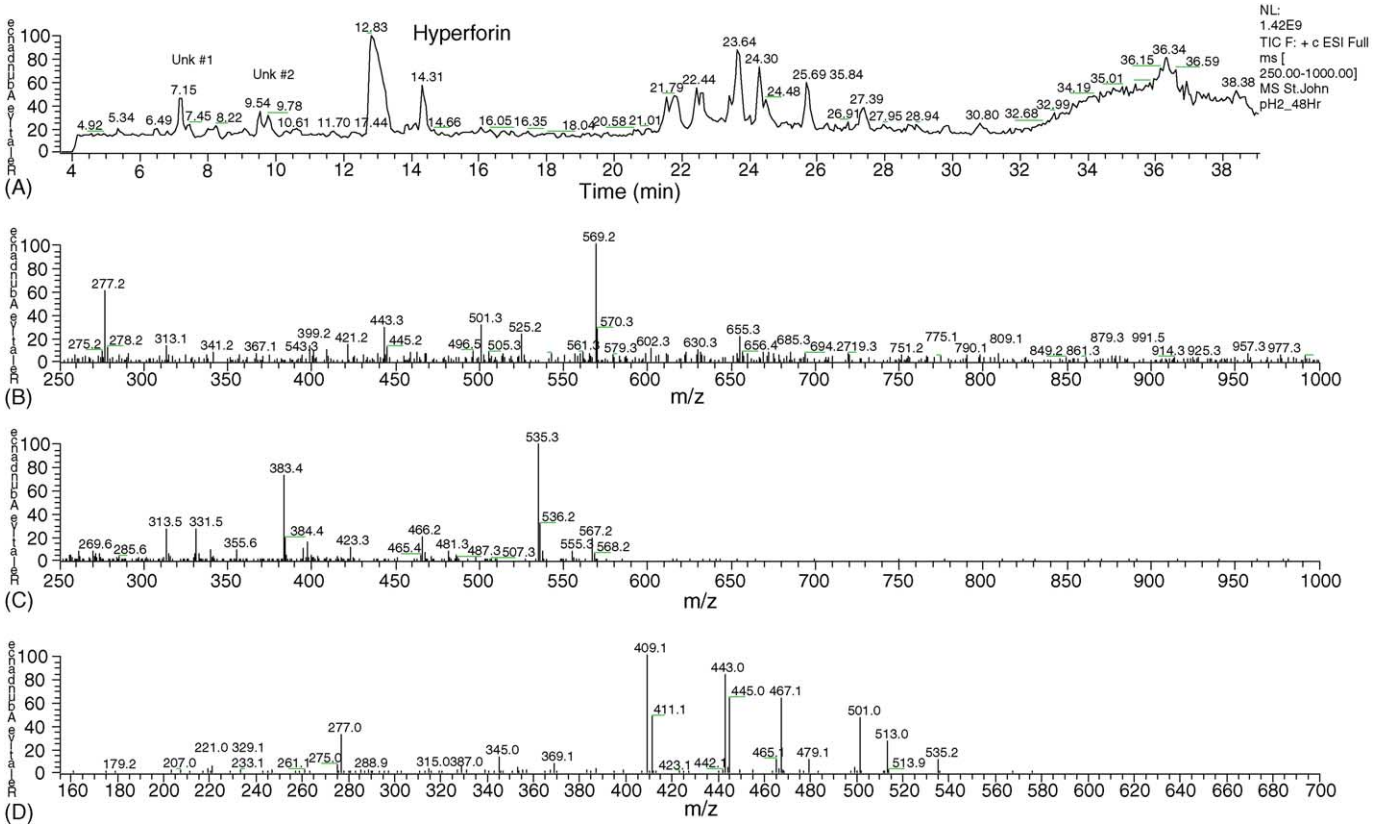


Fig. 7. (A) Total ion (+) chromatogram of St. John's wort methanol extraction at pH 2, light exposure for 48 h; (B) positive ion mass spectrum of the peak at 7.2 min; (C) negative ion mass spectrum of the peak at 7.2 min; (D) positive ion MS/MS spectrum of *m/z* 569.

four naphthodianthrone compounds were observed in the chromatogram. Chromatogram B is the same solution but exposed to light for 6 h. It was noted that hypericin and pseudohypericin (closed-ring structure) are increased at the expense (reduction) of protohypericin and protopseudohypericin (protocompounds, open-ring structure). Chromatographic comparison of these two extraction samples clearly shows that the ring formation is the principal transformation of the 'proto-compound' after light exposure.

It is interesting to note that light exposure has distinctly different effects on hyperforin and hypericin in pH 7 solutions. Hyperforin was seen to decompose completely in 12 h (Fig. 1c) while under the same conditions, hypericin remained stable for 48 h (Fig. 2c). This suggests that different reaction pathways may be occurring for each of these compounds.

The marked increase in oxidation when hyperforin is exposed to light, particularly at neutral pHs, may indicate a susceptibility to light-induced free radical reactions. This observation is consistent with previously published papers on the photochemical characterization of hyperforin and hypericin [20–28]. In contrast to hyperforin, hypericin (closed-ring structure) increased initially through light-induced ring closure of protohypericin (open-ring structure) at neutral pH. The resulting closed-ring molecule is highly conjugated and not susceptible to light-induced free radical reactions. This is consistent with the results in Fig. 2c. For the same reason, similar behaviors were also observed for pseudohypericin and protopseudohypericin (Fig. 3c).

3.2. MS identification of a hyperforin transformation product

In order to identify the structures of hyperforin transformation products using LC–MS, the ion fragmentation pathway of the hyperforin protonated molecular ion ($[M+H]^+$ at m/z 537) was studied. The mass spectra were acquired in both positive and negative ionization modes to determine the molecular weights of the unknowns. In addition, MS/MS spectra were acquired for the selected ions of interest. As shown in Fig. 5, the hyperforin molecule has a unique neutral loss pattern of alkenes. The ion fragmentation in the MS/MS process yields neutral losses of isobutene (−56), isoprene (−68) and dimethylketene (−70). The sub-structures of the hyperforin fragments and the proposed fragmentation pathways are shown in Fig. 6. The presence of these neutral losses in the MS/MS spectra of the selected $[M+H]^+$ ion is strongly indicative of a compound structurally related to the hyperforin molecule.

The peak eluted at 7.2 min was not initially observed in the methanol extractions of St. John's wort. The peak grew over time under light and acidic conditions but did not form at neutral conditions. The unknown has a protonated molecular ion $[M+H]^+$ of m/z 569, confirmed by the presence of a deprotonated molecular ion $[M-H]^-$ of m/z 567. The fragment pattern of this molecular ion (Fig. 7) corresponds well to that of hyperforin (Fig. 5). A comparison of the key fragments of these two compounds is shown in the table below:

Hyperforin	Unknown at 7.2 min	Difference (Da)
537 $[M+H]^+$	569 $[M+H]^+$	32
481	513	32
469	501	32
411	443	32
345	345	0
277	277	0

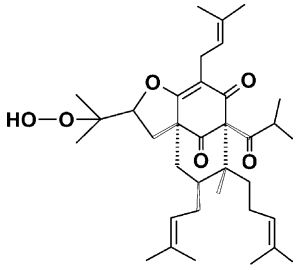
The fragment ions of m/z 277 and 345 are not changed as these two fragments correspond to the hyperforin six- and eight-membered rings, respectively (Fig. 6). Three fragments from the unknown were 32 amu higher than the corresponding fragments from hyperforin. This indicates that these three fragments are structurally related to the hyperforin fragments with the addition of two oxygen atoms.

Further analysis of the mass spectral data reveals five pairs of ions with a common neutral loss of 34 amu in the MS/MS spectrum (Fig. 7), indicating that these two oxygen atoms must be bound together on the molecule since they are lost together. These five pairs of ions are summarized in the table below:

Fragment ions of m/z 569, an $[M+H]^+$ ion of unknown peak at 7.2 min		Difference (Da)
569	535	34
513	479	34
501	467	34
445	411	34
443	409	34

The presence of a neutral loss of 34 amu typically indicates the removal of a hydrogen peroxide (HO–OH) molecule from the corresponding parent ion.

The five pairs of ion fragments strongly suggest that the unknown at 7.2 min is a hydroperoxide compound degraded from the hyperforin molecule. Based on the ion fragmentation pattern discussed above, this unknown is proposed as furohyperforin hydroperoxide and its structure is shown below.



MW : 568.3764
MF : C35H52O6

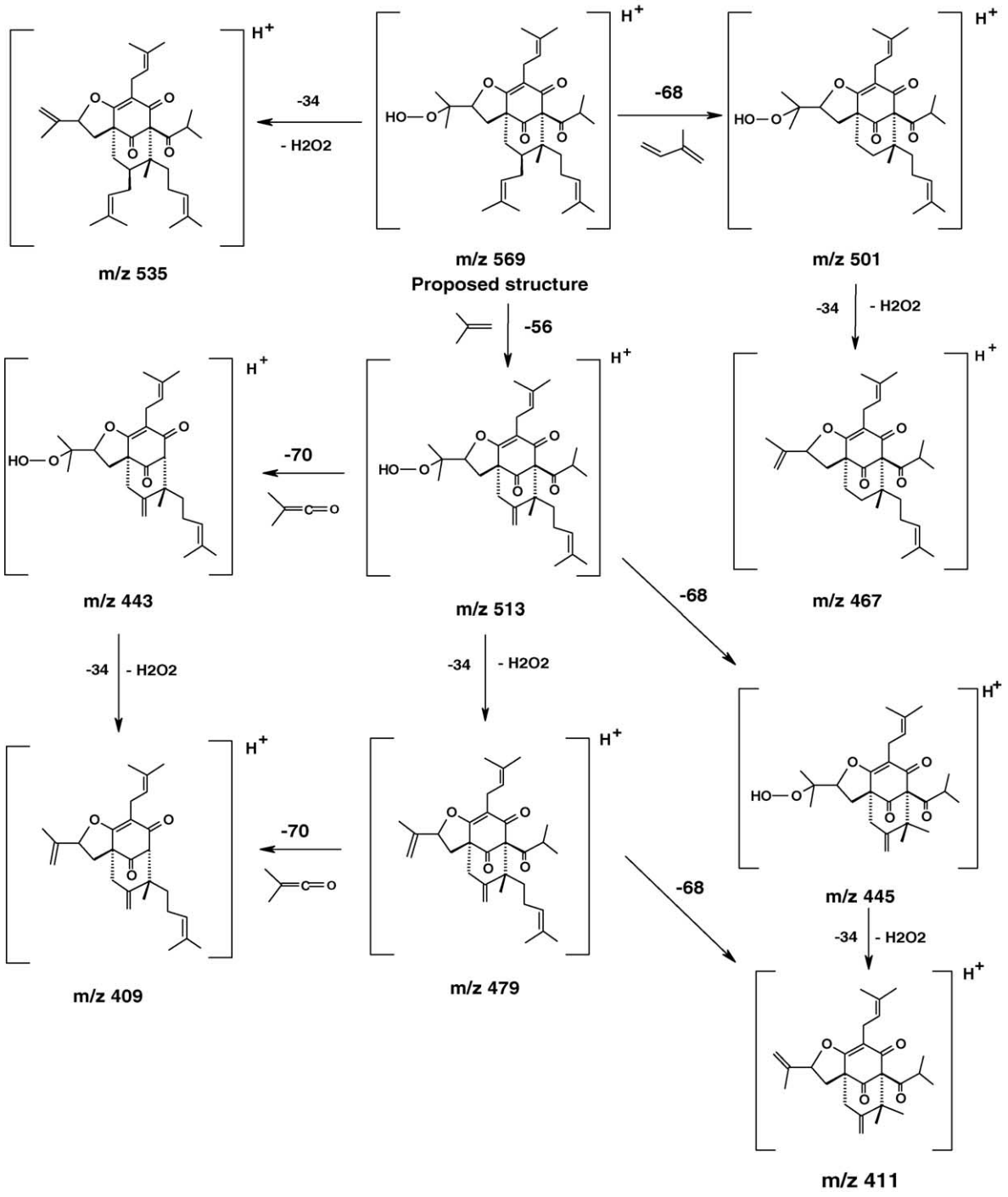


Fig. 8. Proposed fragmentation pathway of *m*/*z* 569 (RT = 7.2 min) for furohyperforin hydroperoxide.

The proposed fragmentation pathway for furohyperforin hydroperoxide is shown in Fig. 8. In addition to the unique neutral loss of alkenes similar to the hyperforin molecule, this molecule shows a unique neutral loss of hydrogen peroxide in five pairs of fragments. The proposed structure matches well with the fragmentation ions detected in the MS/MS studies.

4. Conclusions

Light and solution pH have profound impacts on the stability behavior of methanolic solution of phloroglucinols and naphthodianthrones extracted from St. John's wort powder (*H. perforatum* L.). Under light hyperforin was found to be more stable in an acidic extraction than in a neutral extraction.

LC–MS was used to conduct fast and direct analyses of hyperforin transformation products. One of the hyperforin transformation products found in acidic and light conditions was studied by LC–MS/MS. The transformation compound generated a very unique ion fragmentation pattern, from which its structure was proposed.

References

[1] H. Orth, C. Rentel, P. Schmidt, J. Pharm. Pharmacol. 51 (1999) 193–200.
[2] H. Rompp, C. Seger, C.S. Kaiser, E. Haslinger, P.C. Schmidt, Eur. J. Pharm. Sci. 21 (2004) 443–451.
[3] G.C. de los Reyes, R.T. Koda, J. Pharm. Biomed. Anal. 26 (2001) 959–965.
[4] A. Poutaraud, A. Lobstein, P. Girardin, B. Weniger, Phytochem. Anal. 12 (2001) 355–362.
[5] H. Orth, P. Schmidt, Pharm. Ind. 62 (2000) 60–63.
[6] A. Jensen, S. Hansen, J. Pharm. Biomed. Anal. 27 (2002) 167–176.
[7] F. Liu, C. Ang, D. Spriinger, J. Agric. Food Chem. 48 (2000) 3364–3371.
[8] I. Rager, G. Roos, P.C. Schmidt, K.A. Kovar, J. Pharm. Biomed. Anal. 28 (2002) 439–446.
[9] A. Tolonen, J. Ussitalo, A. Hohtola, J. Jalonen, Rapid Commun. Mass Spectrom. 16 (2002) 396–402.
[10] N. Fuzzati, B. Gabetta, I. Strepponi, F. Villa, J. Chromatogr. A 926 (2001) 187–198.
[11] R. Pirker, C.W. Huck, C.K. Bonn, J. Chromatogr. B 777 (2002) 147–153.
[12] A. Piovan, R. Filippini, R. Caniato, A. Borsarini, L.B. Maleci, E.M. Cappelletti, Phytochemistry 65 (2004) 411–414.
[13] C. Seger, H. Rompp, S. Sturm, E. Haslinger, P. Schmidt, F. Hadacek, Eur. J. Pharm. Sci. 21 (2004) 453–463.
[14] M. Brolis, B. Gabetta, N. Fuzzati, R. Pace, F. Panzeri, F. Peterlongo, J. Chromatogr. A 825 (1998) 9–16.
[15] L. Verotta, G. Appendino, J. Jakupovic, E. Bombardelli, J. Nat. Prod. 63 (2000) 412–415.
[16] L. Verotta, G. Appendino, E. Belloro, J. Jakupovic, E. Bombardelli, J. Nat. Prod. 62 (1999) 770–772.
[17] S. Trifunovic, V. Vajs, S. Maura, N. Juranic, Z. Djarmati, R. Jankov, S. Milosavljevic, Phytochemistry 49 (1998) 1305–1310.
[18] M. Shan, L. Hu, Z. Chen, J. Nat. Prod. 64 (2001) 127–130.
[19] J.L. Wolfender, L. Verotta, L. Belvisi, N. Fuzzati, K. Hostettmann, Phytochem. Anal. 14 (2003) 290–297.
[20] A.R. Bilia, M.C. Bergonzi, F. Morgenni, G. Mazzi, F.F. Vincieri, Int. J. Pharm. 213 (2001) 199–208.
[21] S. Dumas, J.C. Lepretre, A. Lepellec, A. Darmanyan, P. Jardon, J. Photochem. Photobiol. A 163 (2004) 297–306.
[22] A.B. Uzdensky, D.E. Bragin, M.S. Kolosov, A. Kubin, H.G. Loew, J. Moan, J. Photochem. Photobiol. B 72 (2003) 27–33.
[23] H.Y. Du, M. Olivo, B.K.H. Tan, B.H. Bay, Cancer Lett. 207 (2004) 175–181.
[24] E.J. Hunt, C.E. Lester, E.A. Lester, R.L. Tackett, Life Sci. 69 (2001) 181–190.
[25] P. Agostinis, A. Vantieghem, W. Merlevede, P.A.M. de Witte, Int. J. Biochem. Cell Biol. 34 (2002) 221–241.
[26] B.A. Silva, F. Ferreres, J.O. Malva, A.C.P. Dias, Food Chem. 90.
[27] A. Nahrstedt, V. Butterweck, Pharmacopsychiatry 30 (1997) 129–134.
[28] D. Wheatley, CNS Drugs 9 (1998) 431–440.