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Hypericin and Pseudohypericin. Purity Criteria and Quantitative Determination in Extracts of Saint John's Wort (*Hypericum perforatum*)

Youssef Naït-Si and **Jean-Dominique Fourneron***

Laboratoire des Systèmes Chimiques Complexes, CNRS [UMR 6171] Faculté des Sciences et Techniques de Saint-Jérôme, 13397 Marseille, cedex 20, France

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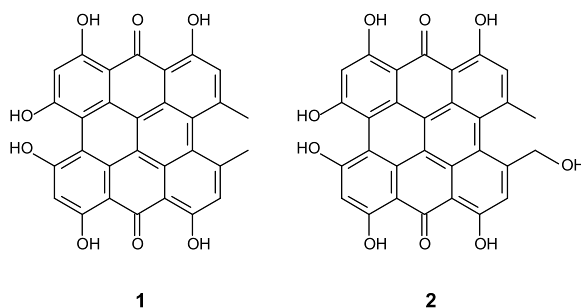
Summary. Hypericin and pseudohypericin in extracts of St. John's Wort (*Hypericum perforatum*) are assayed using UV-Vis, measuring the absorbance at 588 nm of a methanolic solution of the compounds. The value of the absorption coefficient is difficult to define insofar as none of the routine purity criteria can be applied to these two compounds. Analysis of the entire UV-Vis spectrum from 220 to 740 nm would seem to be a reliable way of comparing different samples. A UV-Vis spectrum was recorded for each of the compounds using a Photodiode array detection system. A more accurate way of determining the levels of hypericin and pseudohypericin in extracts of St. John's Wort using these reference spectra is described.

Keywords. Hypericin; Pseudohypericin; Purity criteria; UV-Vis spectroscopy; HPLC-DAD.

Introduction

That extracts of St. John's Wort have biological activity is undeniable, as shown by the use of such extracts in "everyday" medicine ever since ancient times [1]. Today, extracts of St. John's Wort are mainly used in the management of mild depression. The plant has also been shown to have antiviral activity [2, 3] although whether or not it is active against HIV as suggested a decade ago [4–6] is now controversial [7]. Recently, the discovery of the antagonistic activity of the extracts with respect to drugs prescribed for certain major indications (*e.g.* Indavir®, part of AIDS tritherapy, and the immunosuppressive drugs used following organ transplantation) has resulted in health care authorities and the medical community drawing attention to the potential dangers of the uncontrolled use or abuse of this active substance [8–10].

* Corresponding author. E-mail: jean-dominique.fourneron@univ.u-3mrs.fr



Scheme 1

Extracts of St. John's Wort contain numerous compounds, many of which are common in the plant world such as the flavonoids, quercetin, apigenin, bisapigenin, and amentoflavon. However, this small plant which is so common in temperate zones also contains three highly specific compounds, namely hyperforin and the phenanthroperylene quinones hypericin (**1**) and pseudohypericin (**2**). The chemical and physical properties of these have been extensively studied by *Falk* [11].

Some of the extract's various biological activities have been attributed to one or other of these compounds or to synergistic action between them [1], but the antidepressant activity of St. John's Wort is now attributed to hyperforin [12, 13] an unstable C-35 terpenoid. Hypericin and pseudohypericin are both far more stable although pseudohypericin breaks down quickly in alkaline conditions [14, 15].

Given the biological activity of extracts of St. John's Wort, it is important to define a way of gauging their quality. Currently, this is based on measurements of their **1** and **2** content. The reference method is UV-Vis spectroscopy which is relatively simple to perform [16–18]. Both compounds are assumed to have the same absorption coefficient at 588 nm so this method quantitates the two in combination. The two phenanthroperylene quinones **1** and **2** can subsequently be distinguished by liquid chromatography, which provides additional information concerning the quality of a given extract [19]. Each method is associated with advantages and disadvantages. UV-Vis spectroscopy requires knowledge of the specific absorption coefficient. This coefficient is much more stable than the HPLC response factor or retention time and can be considered as a physicochemical property of the compound of the same order as its melting point or specific rotation; it can therefore be used to gauge purity. The absorption coefficient needs to be measured once and for all under defined conditions (solvent, temperature, concentration range, *etc*.). For HPLC, it is necessary to have enough of the stock compound to establish a standard curve. Moreover, since the response factors of **1** and **2** depend on run conditions, regular calibration of the apparatus is essential which means having available samples of the compounds with a known purity. This is a problem when it comes to hypericin and pseudohypericin as long as purity criteria other than the specific absorption coefficient are not taken into account. In summary, both analytical methods require having a pure sample of the substance available at some point, just once for UV-Vis and regularly for HPLC.

This study focuses on the qualification and quantitative determination of **1** and **2** on the basis of analysis of their entire UV-Vis spectra rather than the measurement

of their absorbance at just one or a few wavelengths as is the current norm for assaying extracts of St. John's Wort.

Results and Discussion

*Purity Criteria of **1** and **2***

The signal which we selected as the criterion of purity for **1** was its UV-Vis spectrum between 220 and 740 nm as recorded by a diode array detector (DAD) after injection of methanolic solutions of three different preparations of the compound: that sold by Sigma, another which was kindly given to us by *Falk*, and a third sample prepared in our laboratory [20].

The chromatospectroscopic method [21] was used to check that the peaks observed in the three samples were homogenous, *i.e.* that all the UV-Vis spectra recorded during the appearance of the peak were homothetic with respect to one another. Base-line spectra were identical both before and after the appearance of the peak. In these circumstances, the UV-Vis spectra recorded during the appearance of the peak can be taken as corresponding to pure **1**. Moreover, the elution gradient used means that the spectrum of **1** was effectively recorded in pure methanol. The software Millennium^{32} yields the spectral data (DS) in the form of an Excel®-compatible file which makes it possible to perform comparisons and simple mathematical operations. All three samples tested gave perfectly superimposable UV-Vis spectra. The same solutions were subsequently analyzed with a conventional spectrophotometer over the same range of wavelengths. This instrument too provides the DS in the form of an Excel®-compatible file and again, all three samples tested gave superimposable UV-Vis spectra.

The spectra recorded for the Sigma product using both methods are presented in Fig. 1. Absorption at 588 nm has been adjusted to **1** in both.

The spectrum obtained with the DAD (black line) is less intense, especially below 520 nm. This indicates a higher level of purity as would be expected for a

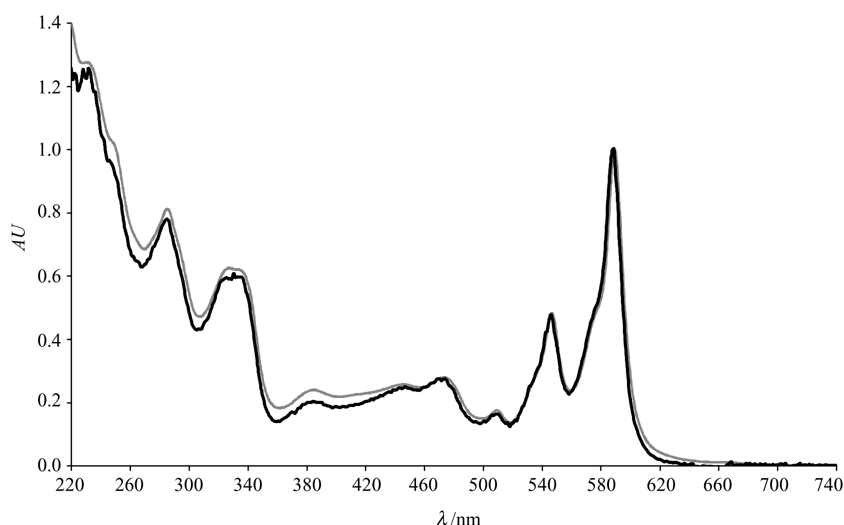


Fig. 1. UV-Vis spectra of **1** (from Sigma) recorded with the DAD (black line) and a spectrophotometer (gray line)

virtual product. Therefore, this trace is considered as being representative of pure hypericin.

For the purposes of quantitative determination, the UV-Vis spectrum can be used to calculate values for $E^{1\%}_{1\,\mathrm{cm}} = 850 \pm 10$ for all three samples. The value used by the German, Swiss, and European Pharmacopoeias [16–18] is 870. Therefore, the purity of the samples is greater than 95%. *Falk* [22] gives a value of $\varepsilon = 43600$ which corresponds to $E^{1\%}_{1\,\mathrm{cm}} = 865$.

Recently, a value of 1025 from $E^{1\%}_{1\,\mathrm{cm}}$ was derived for **1** by *Wirz* [23] but no purity criteria were given for the sample used. The limited UV-Vis spectral data given in this publication suggest that there are no marked differences between the spectra and those presented in this study. This high value does not seem to us to be correct. A possible explanation for these significant differences in the value of $E^{1\%}_{1\,\mathrm{cm}}$ is related to the fact that **1** is relatively insoluble in methanol. Given that the quantities being used are of the order of 1 or 2 milligrams per 100 cm^3 of solvent, visual checking is unreliable and it may be that impurities which dissolve more efficiently are being artifactually over-represented in the resultant solution. To overcome this problem, *Wirz* included pyridine (1% in methanol) to promote the dissolution of **1**. We used *DMSO* (at the same concentration) after checking that it did not affect absorption by **1**.

According to *Falk* the UV-Vis spectra of **1** and **2** are identical in terms of both profile and intensity [19] with ε values of 43600 for **1** and 43100 for **2**. *Wirz* obtained a lower value for **2** ($\varepsilon = 43486$, $E^{1\%}_{1\,\mathrm{cm}} = 836$). Pure samples of **1** and **2**, prepared in our laboratory [20], were analyzed by both HPLC and spectrometer. The chromatospectroscopic method [21] showed that the **1** and **2** peaks were homogenous. The UV-Vis spectra obtained for **2** with the DAD and the spectrometer are shown in Fig. 2.

As precedently with **1**, the spectrum obtained with the DAD is slightly less intense than that recorded by the spectrometer.

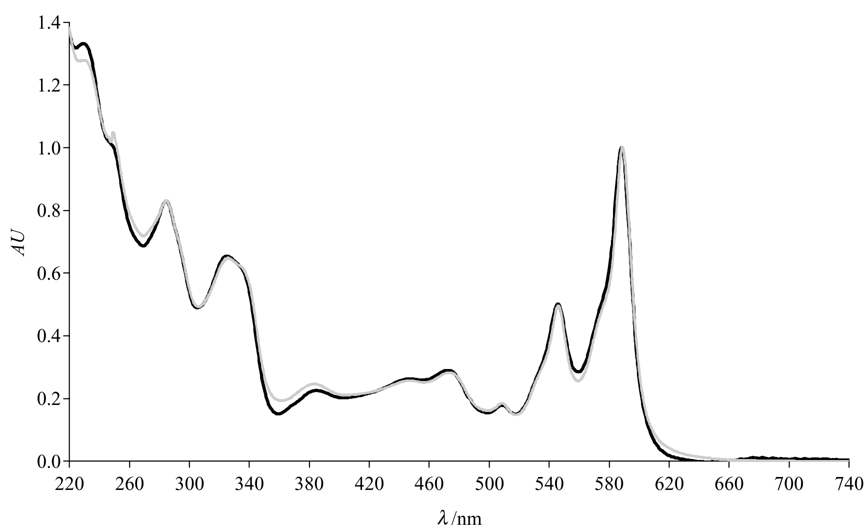


Fig. 2. UV-Vis spectra of pure **2** obtained with the DAD (black line) and with a spectrometer (gray line)

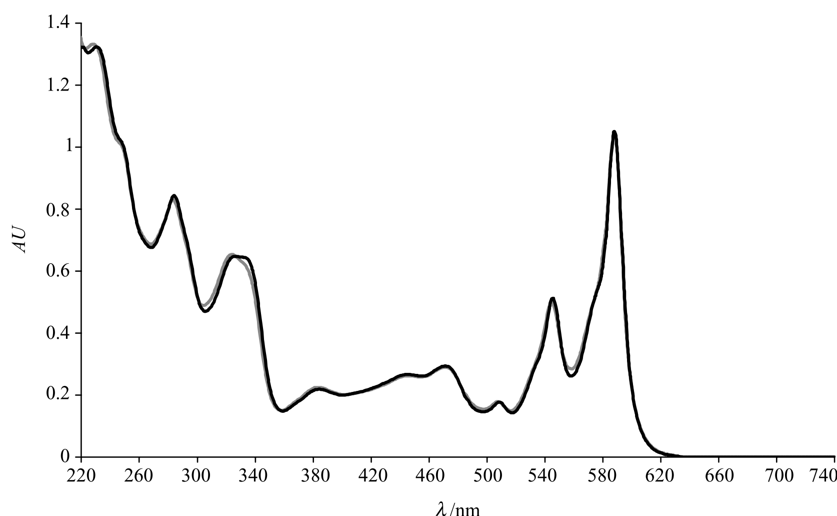


Fig. 3. UV-Vis spectra of **1** (black line) and **2** (gray line) obtained with the DAD

Next, we compared the spectra for **1** and **2** obtained with the DAD. They are shown in Fig. 3. The spectra obtained with the spectrometer are similar to those and are not given.

The samples of **1** and **2** prepared in our laboratory gave similar spectra with both methods. The measured values of $E^{1\%}_{1\,\mathrm{cm}}$ with quantities of between 1 and 5 milligrams were 860 for **1** and 875 for **2**.

On the basis of this similarity, we have assumed – as did *Falk* [22] – that **1** and **2** have identical UV-Vis spectra, in terms of both profile and intensity. The only difference which we systematically observed was between 300 and 360 nm, although this difference was so small that it is difficult to express numerically.

On intuitive grounds, we find it difficult to conceive that two substances with such similar UV-Vis profiles across such a wide range of wavelengths could possibly have absorbance properties as markedly different as those estimated by *Wirz* [23].

Considering the experimental conditions in which **1** and **2** are obtained, notably the low-*pH* precipitation step, it is likely that the two phenanthroperylenequinones are obtained in their protonated form and not in their natural form, *i.e.* potassium and sodium salts [24]. In a polar medium, **1** and very probably **2**, are dissociated [11, 25] and the UV-Vis spectra obtained with the spectrometer are those of its dissociated forms. The similarity of spectra obtained with the DAD implies that **1** and **2** are also eluted and detected in this form.

If the entire UV-Vis spectrum for **1** and **2** is to be used as a purity criterion, it would seem wise to make the spectral data obtained with the DAD and spectrometer available to the scientific community as a whole for reference purposes. These can be found on the Internet at the following URL: http://www.geocities.com/sjwsclub/.

Quantitative Determination of 1 and 2 in Extracts of St. John's Wort

Quantitative determination of **1** and **2** in extracts of St. John's Wort is important because their concentration is a European Pharmacopoeia-approved modality for

assessing the quality of extracts to be sold to and consumed by the general public. In our experience, there is a big difference in the concentrations of **1** and **2** as measured by HPLC and by UV-Vis, with HPLC often giving a significantly lower reading.

A crude extract of St. John's Wort usually contains about 1% of **1** and **2** but, in a significant number of cases, the level is below 0.5%. Of course, this means that 99–99.5% of the extract consists of other substances, including many which are known and recognized as having biological activity.

Assaying **1** and **2** in a dry extract usually involves UV-Vis with a value of $E^{1\%}_{1\,\mathrm{cm}} = 870$ for the absorbance of **1** and **2** at 588 nm [16–18]. Inspection of the UV-Vis spectrum of any crude extract shows that, at this wavelength, **1** and **2** are not the only compounds which are absorbing. Thus, accurate quantitative determination necessitates measurement of absorbance of these impurities. Figure 4 shows the UV-Vis spectrum between 750 and 450 nm of a typical ethanolic extract of St. John's Wort generated from a sample of the raw material used in industrial-scale processes.

The spectrum related to pure **1** or **2** is extracted in the following way: a spectrum of pure **1** or **2** is used as the standard and multiplied by an arbitrarily chosen factor *P*. The resultant spectrum is then subtracted from the spectrum of the crude extract to break it down into two components, one corresponding to pure **1** (or **2**), and another "residual" spectrum corresponding to the absorption profile of the matrix. The absorbance reading at 588 nm on the extracted spectrum then gives the real concentration of **1** and **2** in the extract.

Figure 4 shows three residual spectra corresponding to the factor *P* set at respectively, 0.2, 0.25, and 0.3. Also shown is the spectrum of **1** which was subtracted with $P = 0.25$ (the value which gave the most visually pleasing result). If a value of 0.472 is used for the absorbance at 588 nm, the crude extract contains a concentration of 0.54% of **1** and **2**. If $P = 0.2$, the concentration is 0.23%, and if

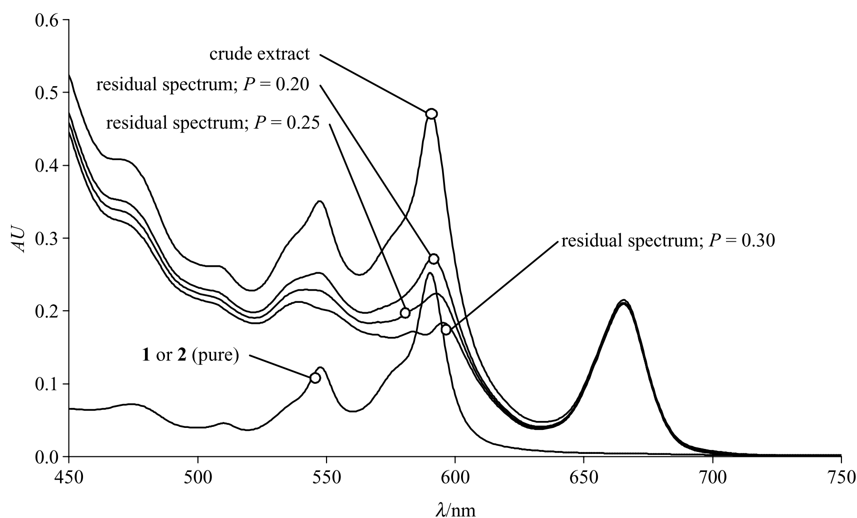


Fig. 4. Crude St. John's Wort extract; extraction of the spectrum for pure **1**

$P = 0.3$, it is 0.35%. When P is set at 0.25, the concentration in the extract is 0.29% in the place 0.54% found without correction. Although this correction entails a subjective step, it nevertheless gives a far more accurate estimation of the concentration of **1** and **2** in extracts of St. John's Wort, in which case the UV-Vis result correlates well with the HPLC result.

The residual spectrum is a mathematical entity. In the course of the work on purifying **1** and **2** [20], various different fractions were obtained from crude extracts which gave information on the absorption profile observed in residual spectra. The peaks definitely correspond to chemical species – not all identified but none the less real.

The method was applied to assaying **1** and **2** in a series of commercially available products, raw materials and formulations. Only some of them were found to contain the claimed concentration of hypericin and pseudohypericin.

Given the differences between **1** and **2** in terms of both stability [14, 15] and pharmacokinetic properties [26, 27] quantifying these two species separately in extracts of Saint-John's Wort becomes indispensable. Hence the two methods, UV-Vis spectroscopy and HPLC, both need to be used. The first one gives an accurate estimate of the concentration of both **1** and **2** in the extract as long as the procedure described is applied and a reference spectrum of the standard compound is used for quantitative determination by HPLC. HPLC analysis gives the relative amounts of **1** and **2** present, as well as their concentrations, on the basis of an external calibration using the standard compound. UV-Vis spectra of the standard compound obtained with a spectrometer and the DAD should be superimposable and the values obtained by the two methods should be consistent.

Experimental

UV-Vis Analysis

Solutions were made up in volumetric flasks using graduated (two-mark) pipettes. Weighing operations were carried out on a Mettler balance to an accuracy of 1/100 mg. UV-Vis spectra were recorded on a Kontron 922 instrument using 1 cm SiO_2 cuvettes. The resolution was 1 nm and the wavelength scanning speed was 100 nm/min.

HPLC Analysis

HPLC was performed on a Waters chromatograph with an E600 pump and a photodiode array detector DAD 996. The unit was controlled using Millennium^{32} software from Waters. Solutions were injected *via* a Rheodyne valve with a 20 mm^3 injection loop. The column (reference: CC125/4 Nucleosil 100-5 C18) was obtained from Macherey-Nagel. DAD settings were: wavelength from 220 to 740 nm; recording frequency: 1 spectrum/s with a resolution of 1.231 nm. The signals generated by the DAD were processed by Millennium^{32} software. Elution was performed at room temperature (20–21°C) in the following steps: $t = 0$, $MeOH/H_2O = 50/50$; $t = 3$ min (linear gradient), *Me*OH; $t = 10$ min (isocratic phase), *Me*OH; $t = 10.1$ (return to initial conditions), $MeOH/H_2O = 50/50$; $t = 15$ min (stabilization), $MeOH/H_2O = 50/50$.

UV-Vis spectra related to a given peak are obtained by using the function "Spectrum Points" of Millenium^{32} giving the spectral data in a compatible Excel® file.

*Purification of **1** and **2***

The purification of **1** and **2** is detailed in Ref. [20]. Briefly, the plant, cultivated by PMA28 Varize, France, is extracted by 20% aqueous acetone. After filtration the liquid extract is defatted by liquid–liquid extraction using *n*-hexane. The aqueous phase is exposed to sun light in order to transform proto-derivatives into **1** and **2** and dried under vacuum. The crude extract is submitted to low-pressure chromatography on C-18 phase (Chromabond® from Macherey-Nagel) with a *Me*OH-H_2O gradient. Fractions containing pure **1** and **2** were pooled. On acidification by addition of 1% H_3PO_4 a black precipitate was collected by centrifugation and washed several times with distilled H_2O and dried under vacuum.

References

[1] Wichtl M, Anton R (1999) Plantes thérapeutiques. Tec & Doc Eds. Paris, p 280
[2] Lopez-Bazzochi I, Hudson JB, Towers GHN (1991) Photochem Photobiol **95**: 54
[3] Cohen PA, Hudson JB, Towers GHN (1996) Experientia **180**: 52
[4] Lavie G *et al.* (1989) Proc Natl Acad Sci **5963**: 86
[5] Meruelo D, Lavie G, Lavie D (1988) Proc Natl Acad Sci **5230**: 85
[6] Schinazi RF, Chu CK, Babu JR, Oswald BJ, Saalmann V, Cannon DL, Eriksson BF, Nasr M (1990) Antiviral Res **265**: 13
[7] Gulick RM, McAuliffe V, Holden-Wiltse J, Crumpacker C, Liebes L, Stein DS, Meehan P, Hussey S, Forcht J, Valentine FT (1999) Ann Intern Med **510**: 16
[8] Turton-Weeks SM *et al.* (2001) Progr Transplant **116**: 11
[9] Barnes J, Anderson LA, Phillipson JD (2001) J Pharm Pharmacol **583**: 53
[10] Schulz V (2001) Phytomedicine **152**: 8
[11] Falk H (1999) Angew Chem Int Ed **316**: 638
[12] Chatterjee SS, Noldner M, Koch E, Erdelmeier C (1998) Pharmacopsychiatry [Suppl 1] **7**: 31
[13] Muller WE, Singer A, Wonnemann M (2001) Pharmacopsychiatry [Suppl 1] **S98-102**: 34
[14] Fourneron J-D, Herbette G, Caloprisco E (1999) C R Acad Sc Série II, 127
[15] Fourneron J-D, Naït-Si Y, Rosas R, Faure R, Viant P (2003) Tetrahedron Lett **44**: 6285
[16] German Drug Codex. St John's wort Monograph. DAC 1986 (3rd Suppl) Govi- and Deutscher Apotheker-Verlag. Eschborn, Stuttgart 1991
[17] Pharmacopoeia Helvetica (Ph Helv 8). St. John's Wort Monograph. Bern. Eidgenössische Drucksachen- und Materialzentrale
[18] European Pharmacopoeia (Ph Eur III Suppl 2000). Hypericum Monograph. Strasbourg, Cedex, Council of Europe
[19] Gaedcke F (1997) Dtsch Apoth Ztg **3753**: 137
[20] Naït-Si Y (2002) Thesis. University of Aix-Marseille III
[21] Fourneron J-D (2001) J Chromatogr Sci **160**: 39
[22] Falk H, Schmitzberger W (1992) Monatsh Chem **731**: 123
[23] Wirz A, Meier B, Sticher O (2001) Pharmazie **52**: 56
[24] Falk H, Schmitzberger W (1992) Monatsh Chem **123**: 731
[25] Leonhartsberger JG, Falk H (2002) Monatsh Chem **133**: 167
[26] Staffeldt D, Geriatr J (1994) Psychiatry Neurol [Suppl 1: S] **47**: 7
[27] Kerb R, Brockmöller J, Staffeldt D, Ploch M, Roots I (1996) Antimicrob Agents Chemother **2087**: 40

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