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***Original Contribution***

# SEPARATE ISOLATION OF HYPERFORIN FROM HYPERICUM PERFORATUM (ST. JOHN'S WORT) PURSUANT TO THE COEFFICENTS LOG $K_{ow}$, $PK_a$ AND DENSITIES OF THE INCLUDED COMPOUNDS

**V. Hadzhiiliev[1]*, D. Dimov[2]**

[1]Medical Faculty, Trakia University, Stara Zagora, Bulgaria
[2]Alfarma, Plovdiv, Bulgaria

**ABSTRACT**
It was proven that the most important ingredients of St John's wort-hyperforins and hypericins have a row of physiological activities. On the base of the preliminary observations that hyperforins are stable at slightly alkaline conditions, on the one hand, and when extracted by a highly non-polar solvents a process of their isolation with a high yield was developed. On the other hand, by having in mind the partition coefficients in octanol water, which are very different for hyperforins and hypericins, the two groups of compounds in a pure form for each one were isolated.

**Key words:** St.John's wort, Hyperforin, Hypericin, Antidepressant and anxiolytic activity

## INTRODUCTION

Hyperforin's structure was established in 1975 in the Shemyakin Institute of Bio-organic Chemistry (USSR Academy of Sciences in Moscow) as a prenylated acyl phloroglucinol (1, 2). Since then many pharmacologic activities of the pure compound were investigated. The main effects of hyperforin are antidepressant and anxyolitic by action as a reuptake inhibitor of monoamines-serotonin, norepinephrine and dopamine and less of other neurotransmitters (3, 4, 5). It was found that it is an inhibitor of cyclooxygenase-1 and 5-lipoxygenase which is 3-18 times stronger than aspirin (6). It also showed antibiotic activity against methicillin –resistant strains (MRSA) (7) and *in vitro* antimalarial effect (8). *In vivo* evidence suggests its efficacy against Alzheimer's disease (9, 10). It is found that hyperforin probably has anticancer (11) and anticlastogenic effects (12). Though the total enantiomeric synthesis was disclosed (13) the main source of hyperforin is St. John's wort yet. The chemical constitutions of St. John's wort are as follow (14, 15): Flavonoids (e.g., epigallocatechin, rutin, hyperoside etc), Phenolic acids (e.g., chlorigenic acid, caffeic acid etc.). Apart from that Naphthodiantrones (e.g., hypericin, pseudohypericin, protohypericin, protopseudohypericin), Phloroglucinols (e.g.,hyperforin, adhyperforin). Some other valuable components also included like tannins, Volatile oils (e.g., 2-methyloctane, nonane, pinenes etc.), Saturated fatty acids (e.g., myristic acid, palmitic acid, stearic acid etc.), Alkanols (e.g.,1-tetracosanol, 1-hexacosanol etc.), Vitamins & their analogues (e.g., carotenoids, choline, nicotinamide etc.) Sesquiterpenes, Chlorophylls etc. Another miscellaneous components also present e.g., pectin, hexadecane etc.

***Correspondence to**: *Dr. Vasil Hadzhiiliev, Trakia University, Med. Faculty, Stara Zagora, Bulgaria, vashi@abv.bg*

## THEORY OF THE WORK

It is well known that Hyperforin is unstable in the presence of light and oxygen (16). Even in the absence of light and oxygen hyperforin in acidic form in usual extragents destroys in a course of one-two weeks (17). The first report of relatively stable extract was made by Maisenbacher P. in his dissertation work in which he macerated St. John's wort in octyldodecanol (eutanol G) and hyperforin isolated by this method was stable for a period of 0.5 years under argon. Some years later it was found that salt forms of hyperforin are much more stable than a free acid (18). A possible explanation for this is that in absence of light and oxygen, the presence of acidic protons catalyses the separation of ethylene or isoprene analogues from the phloroglucinol part. The purpose of our work is to find the

way to isolate hyperforin by higher yield and the optimal conditions for its storage. So, the idea is to extract hyperforin in an acidic form with a highly non-polar extragent to avoid simultaneous extraction of other (more polar) acidic compounds. This is a possible explanation of the result of Maisenbacher, too. At the same time the residue not dissolved in the highly non-polar solvent could be used for isolation of hypericins, which have many pharmacologic applications, too. In the next table are shown log $K_{ow}$ values for a row of compounds **(Table 1):**

***Table 1.*** *Partition coefficients octanol water*

| **Compound** | **Log $K_{ow}$** | **Reference** |
|---|---|---|
| Hyperforin | 13.17 | (21) |
| Alpha-tocopherol | 11.9 | (19) |
| Hypericin | 8.78 | (20) |
| Pseudohypericin | 7.32 | (20) |
| Flavone | 3.56 | (19) |
| Tetracosane | 12.13 | (20) |
| Octyldodecanol | 9.19 | (19) |
| Dicyclohexylamine | 3.69 | (19) |
| Heptane | 4.47 | (19) |
| Caffeic acid | 1.42 | (19) |
| Tannic acid | -0.19 | (20) |
| Beta-pinene | 4.37 | (19) |
| Palmitic acid | 7.17 | (21) |
| Beta-carotene | 17.62 | (20) |
| Tetracosanol | 10.66 | (21) |
| Chlorophyll A | 16.87 | (20) |

The pKa value for hyperforin was measured 6.32 (22). For the monodeprotonation of hypericin was found $pKa_{,1}$=2 and for dideprotonation $pKa_{,2}$=11(23). For the density of hyperforin in Chemical Book the value of 1.01 was found, no data for hypericins densities were found and for dicyclohexylamine pKa=10,4,density=0,91 and vapour pressure at 37,7 C=12 mm Hg. For the needed in our process paraffin oil (CAS 8042-47-5, a mixture of isoalkanes and cycloalkanes with an average hydrocarbon tetracosane) the density is approx.0, 8.

## MATERIALS AND METHODS

*Hypericum perforatum* dried tops and leaves powdered to approx. 25 mesh; All-rac-alpha-tocopheryl acetate MERCK 500952; Methanol 99.9%; Ammonia 25% analytical grade; Ascorbic acid pharmaceutical grade; Potassium hydroxide analytical grade; Ethanol absolute 99,8%; Citric acid food grade; Sodium chloride food grade; Paraffin oil, chain length C18-C30 EP-6.0; Magnesium sulfate anhydrous analytical grade; Kaolin powder mean particle size 0,65 um (Sedigraph device); Millipore HAWP04700 pore size 0,45 um; Dicyclohexylamine MERCK 802948; Heptane 99%; Calcium hydroxide analytical grade; Calcium chloride anhydrous; Diethyl ether analytical grade; TLC-plates MERCK 105553; Petroleum ether 40-60 C; Acetone analytical grade; Dimethylformamide analytical grade; Glacial acetic acid.

### Description of the process

To 11, 0 g of tocopheryl acetate a solution of 5,0 g potassium hydroxide in 200 mL absolute ethanol is added. The mixture is kept for shaking and stored at ambient temperature for 20 hours. The semiquantitative TLC (see below) shows the conversion to free tocopherol more than 90%. The solution is mixed into 3,5 L methanol and a warm solution of 108 g ascorbic acid in 120 mL water with 25% ammonia are added. The solution is poured in a dark vessel and 500 g powdered *Hypericum perforatum* are added. The mixture was shaken periodically for 10 hours and the first extract is removed from the hard residue by pressing through a piece of mesh cloth. The remaining part of the extract was additionally extracted by 4 portions of totally 7 L methanol with warm solution of 70 g ascorbic acid in 90 mL water and 80 mL 25% ammonia. The whole mixture was filtered through filter paper and a semiquantitative TLC experiment was showed content of approx. 25 g hyperforin and approx. 2 g adhyperforin (sum approx. 50 mmol). The pH value by wet indicator paper is approx. 9,5. Different TLC (see below) shows a presence of red spots. To the solution 500 mL paraffin oil

and a solution of 175g citric acid and 750 g sodium chloride in 4,5 L water are added. The mixture is shaken in a dark vessel for an hour and the layers are separated. The lower layer is additionally extracted by 175 and 2x100 mL paraffin oil. The pH of the methanol-water layer is approx. 6 and the TLC analysis shows no presence of hyperforin. The paraffin oil layer (approx. 1 L) was additionally separated in a dark separate funnel overnight; the small methanol-water layer is discarded and to the oily residue 100 g anhydrous magnesium sulfate is added. After shake for hour 50 g kaolin powder are added and the mixture is shaken for additional 2 hours. After that the mixture is centrifuged at approx. 2500 G for 0,5 hour. To the sediment 1 (approx. 250 g) 2 g ascorbic acid and 200 mL dimethylformamide (DMFA) are added and the mixture was stirred in a dark hermetic vessel for 2 hours and centrifuged at approx. 2500 G for 0,5 hour. The supernatant is additionally separated in a dark separation funnel and the lower DMFA solution is analyzed by TLC. The supernatant of the sediment 1(paraffin oil solution) is filtered through Millipore filter (0,45 um) and to the filtrate 15 mL dicyclohexylamine (75 mmol) are added. The mixture is shaken and stored at 0°C for two days. After that the suspension is centrifuged at. approx. 2500 G for 0,5 hour. The sediment 3 weights 53 g and to it 300 mL heptane, 7 mL absolute ethanol and 7 mL dicyclohexylamine are added. The mixture is intensively stirred for 0,5 hour and put to stay at 0°C for two days and at -18°C additional two days. The precipitate is filtered off and the cake is washed by total a mixture of 50 mL heptane,1,2 mL absolute ethanol and 1,2 mL dicyclohexylamine chilled to -18°C. The cake was put into in advance prepared hermetic dark vessel, containing separated paraffin oil, calcium chloride anhydrous, a mixture 1:1 w/w of calcium hydroxide and ascorbic acid and a small amount of dicyclohexylamine. The reason is that paraffin oil absorbs the heptane vapors, calcium chloride absorbs traces of water and ethanol, the mixture 1:1 removes oxygen and carbon dioxide, hence dicyclohexylamine prevents the dissociation of the hyperforin dicyclohexylammonium salt. After 5 days at ambient temperature the dry pale green crystalline powder weighted 26,5 g (43 mmol). Melting point by a device SMP-10 was 165°C as in (18). After storage of the substance in the same vessel at ambient temperature for 6 months there was no change in the melting point and in comparison with a freshly prepared by the same method substance at TLC analysis.

**Analyses**

TLC-1:

Mobile phase: heptane: diethyl ether = 4:1 + 0,5% v/v glacial acetic acid

TLC plates: MERCK 105553

Standard solution: all-rac-alpha-tocopheryl acetate 1% in heptane

Visualisation: iodine vapors 0,5 hour

1. Analysis of tocopherol solution: 20 uL of the standard solution of 2,0 uL of the all-rac-alpha-tocopheryl acetate hydrolysate was placed on the plate in a stream of nitrogen and the spots are allowed to separate of its components by approx. 1 cm by mobile phase two times. The chromatogram is developed to at least 10 cm, dried and kept in an iodine vapor camera for 0,5 hour. The $R_f$ values are: for the standard solution 0,55; for the hydrolysate mainly 0,45 and less than 10% at 0,55.

2. Analysis of the methanol extract: 80 uL of methanol extract an 20 uL of the standard solution are placed on the start of the plate under stream of nitrogen and the spots are expanded by mobile phase to approx. 1 cm twice. The chromatogram is elaborated as in point 1 and for the sample the next Rf values were found: traces at 0,55; approx. a half of the standard at 0,45; approx. equal to the standard at 0,36; approx. 10% of the standard at 0,40. Most probably at 0,36 is hyperforin an at 0,40 is adhyperforin. At the start there is a nearly black spot.

3. Analysis of the acidic paraffin oil extract after the first centrifugation and filtration through Millipore filter: 7 uL of the extract and 20 uL of the standard solution are elaborated as in point 1. The chromatogram appears bluish-green as in point 2.

4. Analysis of the substance: 30 mg of the substance are dissolved in 4,0 mL chilled methanol. 40 uL of this solution and 20 uL of the standard solution are elaborated as in point 1. Practically no colored products are present. In the sample there are a spot equal to standard at $R_f$ 0,36 (hyperforin), a spot approx. 5% of the standard at $R_f$ 0,40 (adhyperforin) and a spot at the start point, what is, most probably, dicyclohexylamine acetate.

TLC-2:
Mobile phase: Petroleum ether 40-60°C: acetone: dimethylformamide = 7:3:1 + 0,5% v/v glacial acetic acid;

TLC plates MERCK 105553

40 uL of the methanol extract; 1 uL of the DMFA extract from sediment 1; 4 uL of the acidic paraffin oil extract and 4 uL of the alkaline paraffin oil extract after the third centrifugation are placed on the start of the plate in a stream of nitrogen and the spots are expanded by mobile phase to approx. 1 cm. The plate is developed in mobile phase for at least 10 cm. In the first two samples red spots at $R_f$ 0,62 are visible. This is most probably a mixture of hypericins. In the third and fourth samples no red samples are present only slight yellow spots at $R_f$ 0,66 and dark bluish-green spots at $R_f$ 0,38 are observed.

## RESULTS AND DISCUSSION

1. It was found, that when the dicyclohexylammonium salt of hyperforin is stored at ambient temperature in suitable conditions preventing oxidation and its conversion to an acidic form, it is stable for at least 6 months. This in our opinion show that except of storage on light an oxygen, the acid catalyzed destruction is possible.

2. It was found, that the use of paraffin oil as an extragent is very useful to extract the acidic form of hyperforin without any extraction of other acidic compounds.

This fact, in our opinion, could be explained by very close values of the Log Kow for hyperforin (13, 17) and for paraffin oil (modeled by tetracosane-12, 19) and very different values for hypericin (8) and pseudohypericin (7).The relatively high value of pKa of hyperforin show that the autocatalytic destruction is slightly possible. By the other side carbonic acid (pKa1=3,6 for $H_2CO_3$ only, pKa1=6,3 including $CO_2$(aq)) is enough strong to convert hyperforin dicyclohexylammonium salt to a free acid.

### Future plans

1. In our opinion, observed in (12) anticlastogenic efficacy show that radioproctective properties of hyperforin in similar conditions are very possible.
2. From the sediment 1 could be find the way to isolate hypericins.

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