Acta Veterinaria (Beograd), Vol. 47. No. 5-6, 331–344, 1997.

UDK 619:615.076.7

BIOCHEMICAL REACTIONS IN VITRO OF CERTAIN FUNGI TO THE PRESENCE OF HYPERICIN

SLAVICA SOLUJIĆ*, S. SUKDOLAK,* LJILJANA ČOMIĆ* and LJ. KRSTIĆ,**

*Faculty of Science, Department of Chemistry and Bilogy, Radoja Domanovića 12, 34000 Kragujevac, Yugoslavia
**Center for Chemistry, Institute of Chemistry, Technology and Metallurgy, Njegoševa 12, 11000 Belgrade, Yugoslavia

(Received, 5. September 1997.)

In this work,the biochemical reactions of the fingi A. niger, P. verrucosum, T. viride, T. harzianum and D. stemonitis to the presence of 30 mg/ml, hypericin, added to the standard nutrient base, were investigated. Hypericin acts in two ways either as an activator of biochemical reactions or as an inhibitor, depending on the fungual species.

In already prepared methylene chloride extracts of 14-day old fungi, both the number and the chemical structures of biodegradational products were examined in order to register the possible involvement of hypericin in fungal biochemical reactions and to determine the level of biodegradation. The rate of biodegradation and the number of biodegradational products differed from case to case, with protohypericin as a common intermediary compound.

Hypericin was bactericidal to Streptococcoccus,A.Streptococcus B, Enteroccocus, E. coli and P. mirabilis in the concentrations of 10 mg/ml, and 20 mg./ml.

Key words: Hypericin, microbial degradation, bacteria, fungi

INTRODUCTION

Dissimilation of phenol and other aromatic compounds under the influence of microorganisms, studied by many research scientists, confirmed microbial degradation of the aromatic ring. Biodegradational mechanisms for aromatic compounds, optimal reaction conditions and the chemical structure of intermediary products were reported by Happold (1950). Czekalowski and Skarzynski, 1948, and Kramer and Doetsch 1950. investigated the effects of the starting phenol, i. e. different chemical structures, on the level of degradation by different microbial species such as Achromobacter, Micrococcus and Vibrio.

Degradation of phenol is catalyzed by oxygenases (Neujahr, 1972; Koch and Kruger, 1995.) highly specific enzymes, resulting in the formation of o-hydroxyl products, such as o-diols. Decomposition of the phenolic ring and subsequent degradation proceed in common metabolic pattways, via intermediates, such as acetyl-CoA or succinyl-CoA. Certain differences between the metabolic routes occur with of different microorganisms.

Red hypericin phenol powder (Figure. 1) was obtained from the plant

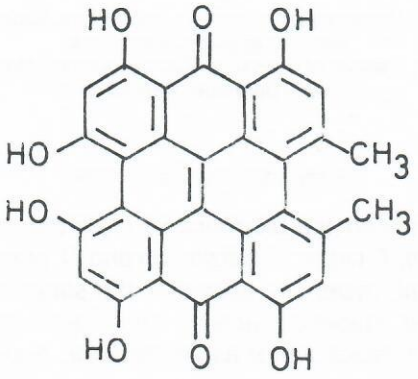

Figure 1. Chemical structure of Hypericin

species Hypericinn perforatum (Helentiae), after acidic hydrolysis of the glycoside (Brockmann and Eggers, 1958)

Hypericin contains two different functional groups: phenolic and quinonic. Such molecules are carriers of biological activity, being efficient antioxidizers and biocatalysts of non-enzymatic oxidoreductions, proceeding by the quinone-hydroquinonic mechanism. Similar biochemical transformations are assisted by the vitamins (A, E, K, C) and different quinones, acting as donors or acceptors of electrons or protons (Bell et al., 1972). Aqueous and oil extracts of the plant Hypericum perforatum are used in medicine as an antidepressant or relaxant.

Many pigments whose structure is similar to hypericin are formed as secondary metabolites of numerous fungi and plants. They are chemical derivatives of emodyne, dermocybine (Birkinskaw, and Grurlay, 1961), skyrine (Shibat a et al. 1968), protohypericine, phagopyrine penicillopsine (Brockmann, and Egger 1958, Brockmann and Neeff, 1951).

The aim of this study was to characterize the effect of hypericin, on biochemical reactions of some fungal cultures. This effect was followed via the change of significant biochemical parameters, such as pH, redox potential, proteolytic activity and fungal bioproduction, between the 4th and 10th day following inoculation.

We also investigated the participation of hypericin in some metabolic reactions of the fungi, depending on their ability to degrade the phenol.

By applying a method of diffusion on agar, we investigated the bactericidal effect of 10 mg/mL and 20 mg/mL hypericin on certain bacteria (Sponer et al., 1972).

MATERIALS AND METHODS

Microorganisms. Monosporal cultures: *A. niger, P. verrucosum, T. viride, T.harzianum* and *D. stemonitis* were obtained from the mykotheque of the Faculty of Natural Sciences, Kragujevac. The cultures of *Streptococcus A, B, Staphylococcus, Enterococcus, Pseudomonas aeruginosa, Escherichia coli, and Klebsiella* were obtained from the microbiological department of the Institute for Hygiene, Kragujevac.

Fermentation. The cultures of fungi were grown on potato-glycose nutrient agar, at 4^0C. Monosporal cultures were obtained by exhaustion on pure agar, in Petri dishes. In our experiment, the fungi were grown on the mineral nutrient base of Czapek which had the following composition (g/L): NaNO3 - 3, K_2HPO_4 - 1, $MgSO_4 \times 7\ H_20$ - 0.50, KCl - 0.50, $FeSO4 \times 7\ H_20$ - 0.01, saccharose - 30, distilled water - 1000 (at pH 7.3).

Hypericin was in the form of a dark red amorphous powder,precipitated after acidic hydrolysis (1:1 HCl) of the ethanol extract of dry crushed plant. This precipitate was filtered, well rinsed and air dried. Its chemical composition, solubility, melting point and chemical structure were determined and found to be in agreement with the data in the literature (Brockmann and Eggers 1958).

The influence of hypericin on metabolic reactions of the fungi was investigated in 100 mL of nutrient base after addition of 30 mg/mL of aqueous hypericin solution and 10 mL of the spore suspension. Incubation was performed at 27^0C, without shaking, under alternate day/night conditions.

Biochemical parameters, pH Change,proteolytic enzyme activity (PE), redox potential (rH_2) and fungal bioproduction were examined between the 4th and 8th day following inoculation, applying standard experimental methods. The proteolytic activity of fungi (PE) in l mL of nutrient base was determined by Anson's method, on the basis of Tyr or Trp formed from casein under the influence of proteases, using the standard, straight-line method (Dudka, 1982). At the end of the experiment, after 14 days incubation, mycelia were separated and the liquid medium extracted with 3 x 50 mL methylene chloride. The extract obtained was concentrated, dried over anhydrous Na_2SO_4 and analyzed thin-layer (TL) chromatography on silica gel, in benzene-ethylacetate (8.5:1.5) The spots were made visible by exposing the chromatogram to iodine vapours. After that, Rf values for hypericin and degradational products were determined.

The bactericidal effect of hypericin on some gram-positive and gram-negative bacteria was determined by a disc diffusion method, on sterile Columbia blood agar. The cultures were incubated for hours, at 37^0C. The level of inhibition was determined after 48 hours development.

RESULTS AND DISCUSSION

Hypericin, added to the standard nutrient base of Czapek in the amount of 30 mg/mL markedly changed the biochemical reactions of *A. niger, P. verrucosum, T. viride, T. harzianum* and *D. stemonitis.* These changes were

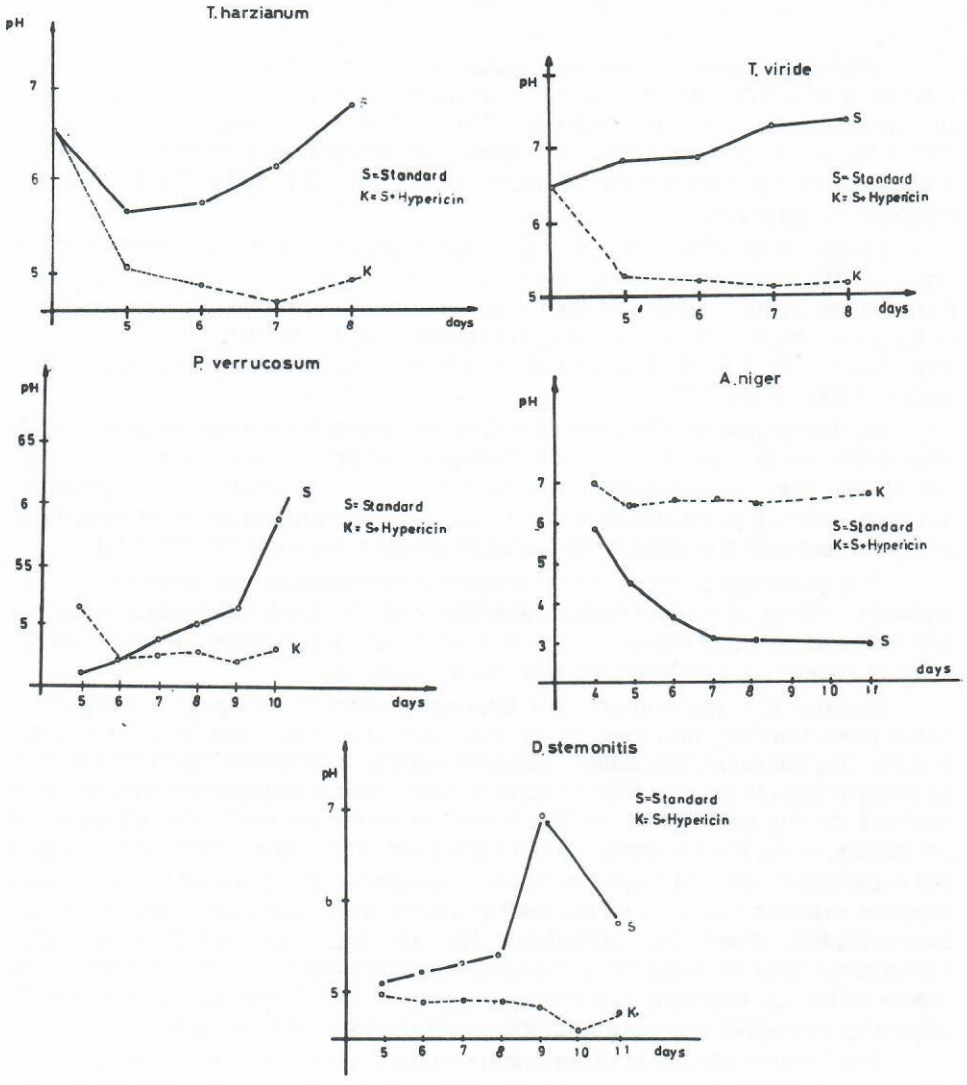

Figure 2. Changes in pH of the standard nutrient base of Czapek with and without hypericin, cultivated with the fungi Trichoderma Doratomyces, Penicillium and Aspergillus

monitored via metabolic parameters, determined between the 4th and 10th day following inoculation of the cultures and are presented in graphic form for each biochemical parameter separately in Figures 2-6.

Change in pH of the nutrient base for the investigated fungi are presented in Figure 2.

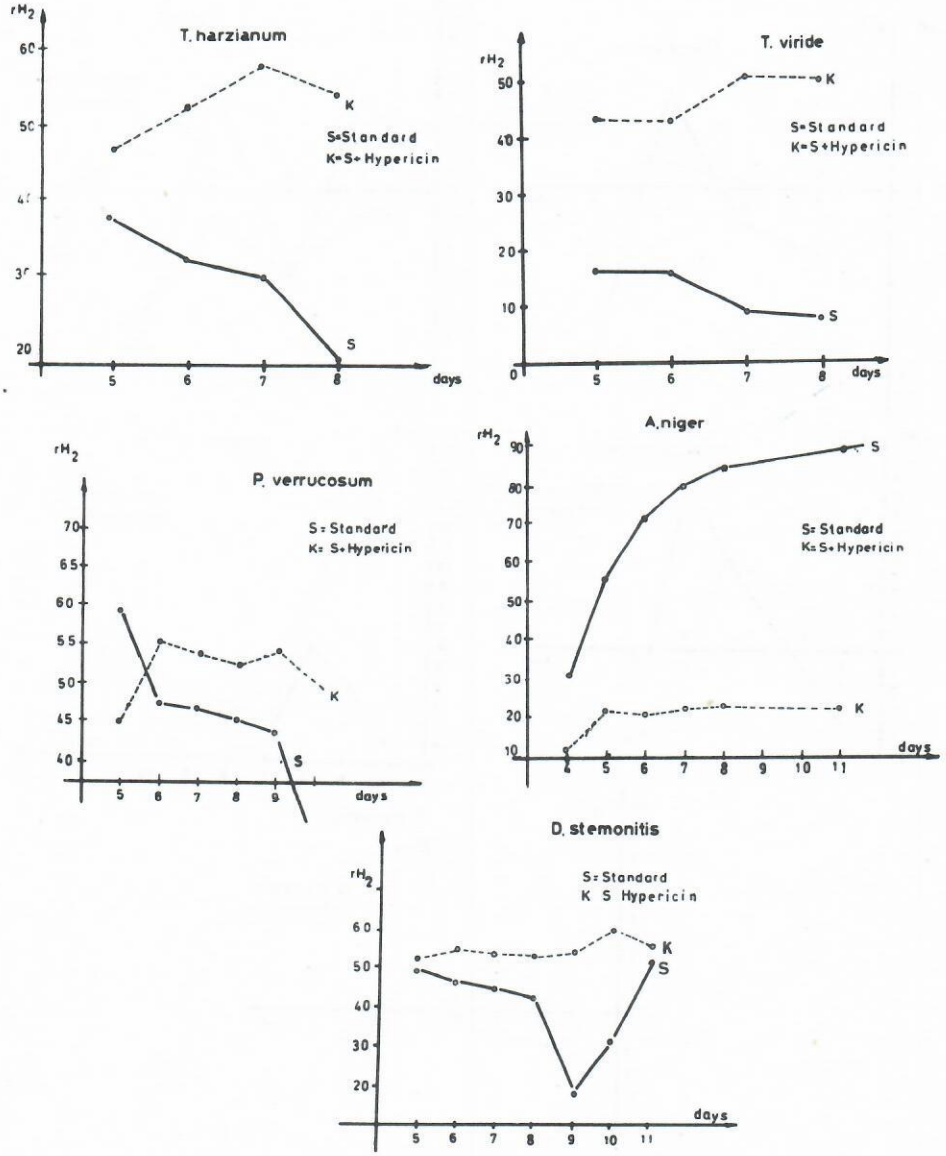

Figure 3. Changes in rH_2 of the nutrient base with and without hypericin for the fungi Trichoderma, Doratomyces,Penicillium and Aspergillus.

In Trichoderma genera, hypericin shifed the pH value to the acid range, reaching the minimum on the 5th day for T. viride, and on the 7th day for T. harzianum.The same trend was repeated for P. verrucosum and D. stemonitis, where the minimum was registered on the 7th, and 10th day, respectively. During

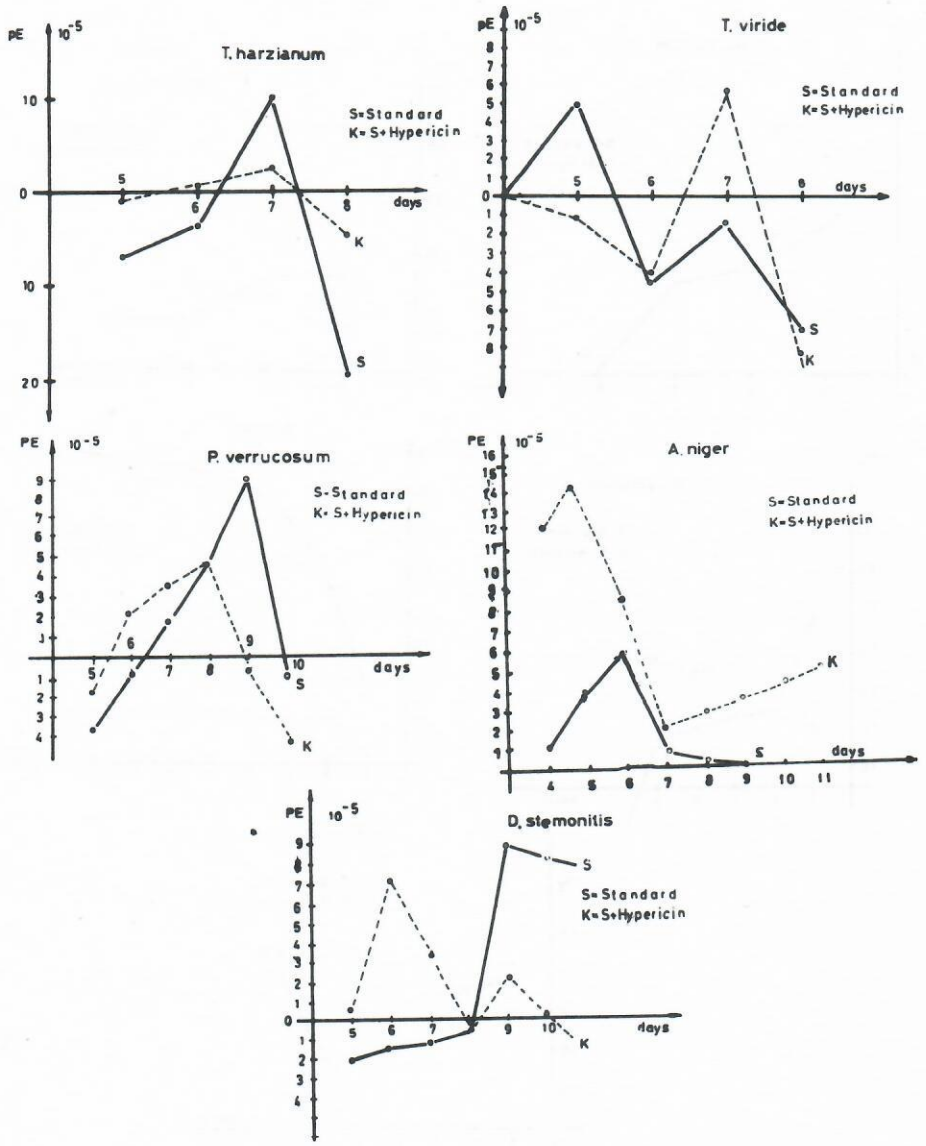

Figure 4. Change of proteolytic enzyme activity (PE) in the standard nutrient base of Czapek, with and without hypericin for the fungi Trichoderma,Doratomyces, Penicillium and Aspergillus

the experimental period, the four species were developing at a nearly constant pH of approximately 5.

The behaviour of A. niger was exceptional, its development proceeding in the presence of hypericin at a nearly constant pH of approximately 6.2 over the entire experimental period.

These results, observed separately, might be explained as follows hypericin noticeably influenced the development of A.niger, while the other fungi successfully adapted to the changed development conditions.

The redox potential of the standard nutrient base of Czapek alone and with the addition of hypericin at 30 mg/mL are comparatively presented for the fungi a. niger,P. verrucosum, T. viride T. hrzianum and D. stemonites in Figure 3.

The presence of hypericin in the nutrient base caused an increase of the redox potential over the entire experimental period for the genera Trichoderma, Doratomyces and Penicillium. Trichoderma showed the maximal potential on the 7th day, reaching a value 2-3 times higher than in the absence of hypericin.

For the species Doratomyces, a slow increase of rH_2 was registered over the entire experimental period while for Penicillium, the maximal measured value, reached on the 6th day, was 10 mV higher.

A specific case was registered for A. niger, where hypericin inhibited oxidoreduction reactions so that the potential of 20 mV was maintained throughout the experiment (Roller et al., 1984.)

The influence of hypericin in the standard nutrient base of Czapek on proteolytic activity of Trichoderma, Penicillium, Doratomyces and Aspergillus is presented in Figure 4.

The activity of proteolytic enzymes of the investigated fungi developing on nutrient base in the presence of hypericin varied, depending on the species (Fukomoto et al. 1967.).

Hypericin was a strong inhibitor of the proteases in Trichoderma genus. T. viride was more vital, exhibiting maximal activity on the 7th day, equal to that reached by the 5 day old pure culture. For Penicillium fungi, the PE curve showed maximal enzymatic activity on the 8th day following inoculation (one day later than with the pure culture) and its value was 40% lower. In the case of Doratomyces, maximal activity was reached on the 6th day (9 days with the pure culture), but this value was still 10% lower than the standard one. The second stage of protease activity was a decreasing trend well expressed between the 8th and 9th day.

The A. niger culture showed raised enzymatic activity over the 7 day development period the maximal PE value, reached on the 5th day (2 days earlier than with the pure culture) was two times higher in comparison with the standard one. The development period, between 5-7 days was characterized by PE decrease, gradually rising after it (Yushido, 1956).

Bioproduction of Trichoderma, Penicillium, Doratomyces and Aspergillus, developing on the changed nutrient base containing 30 mg/ml hypericin, is presented in Figure 5.

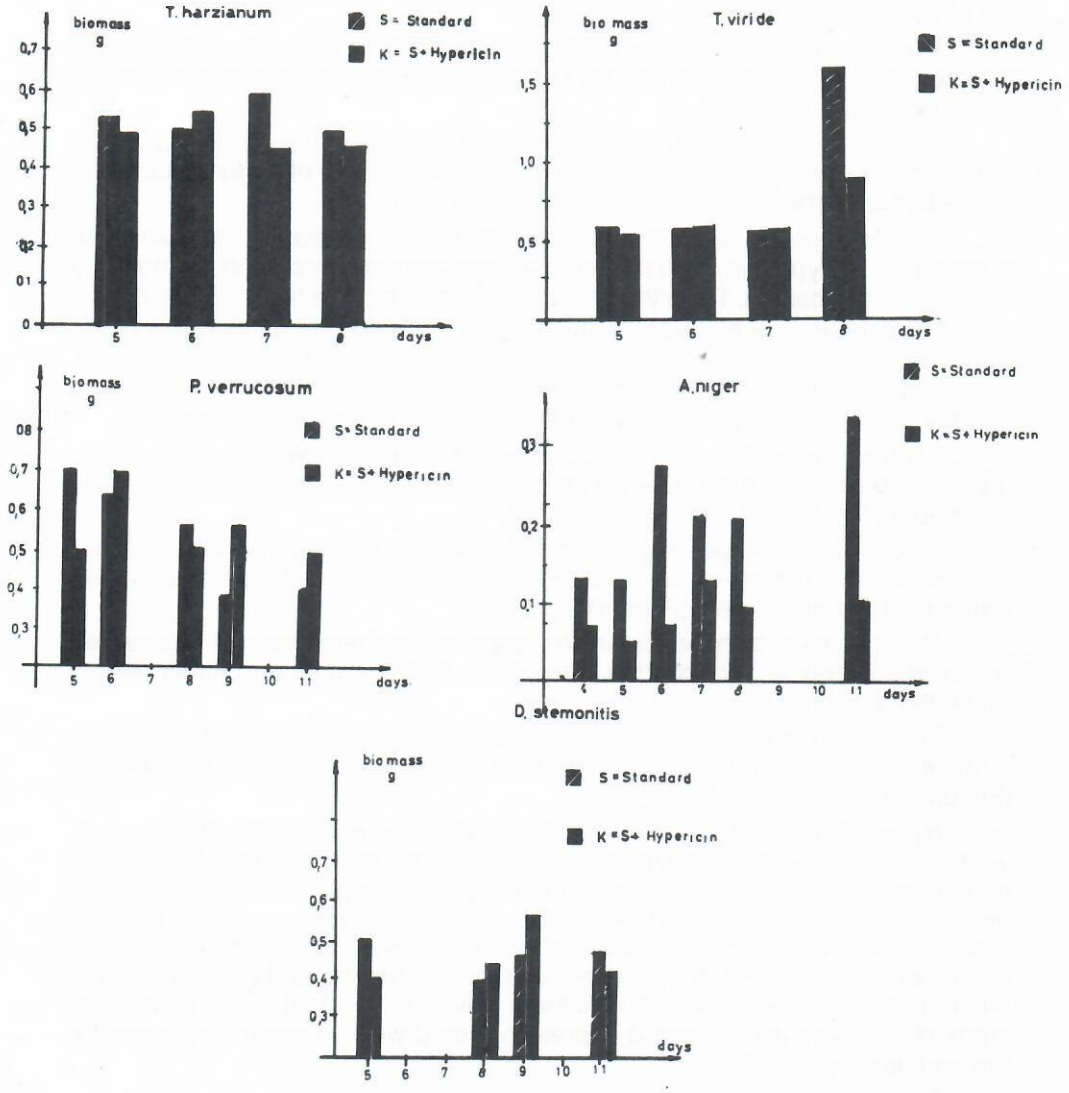

Figure 5. Bioproduction of Trichoderma, Penicillium, Doratomyces and Aspergillus, cultivated on nutrient base with and without hypericin

Hypericin did not have a great influence on bioproduction of Trichoderma fungi. In the case of T. harzianum, a slow increase of the biomass in the presence of hypericin lasted for only 6 days;after that, it was 5-10% lower. T. viride had the same level of bioproduction between 5-7 days, quite independent of hypericin. However, on the 8th day, the biomass was reduced by 40% in comparison

with the pure culture. Development of Doratomyces fungi was slowed until the 8th day, becoming intensified after that, while Penicillium had cycles of intensified and slowed development. An increase in biomass was registered on the 6th, 9th and 11th following inoculation.

For A. niger, hypericin was a strong inhibitor of bioproduction between 4-8 days. Thus, minimal development was registered on the 6th day; when it was about 60% lower than with the pure culture. On the other days, the decrease was in the range of 20-40%.

The level of the hypericin biodegradation by the biochemical reactions of the fungi: *Trichoderma, Penicillium, Doratomyces* and *Aspergillus* after 14 days growth was determined by analyses of the mycelia extract in methylene chloride and the solution (Naumova, 1985, Solujić, et al. 1994). The extract was analyzed by TL-chromatography and R_f values obtained are presented in Table 1.

Table 1. Chromatographic R_f values of some hypericin biodegradational products

Culture	R_f of hypericin	R_f of the metabolites		
Trichoderma viride	0.98	0.84		
Trichoderma harzianum	0.98	0.84		
Doratomyces stemonitis	0.98	0.84	0.60	0.40
Penicillium verrucosum	0.98	0.84	0.65	
Aspergillus niger	0.98	0.84	0.70	0.40

The chromatographic analysis led us to the conclusion that the investigated fungi included hypericin in their metabolic reactions during the 14-day vegetative period, showly degrading it.

Trichoderma cultures produced only protohypericin, while the other fungi produced one or two products more. *Doratomyces* and *Aspergillus* degraded the phenolic molecule to emodyn-9-anthrone. The absorption spectra for hypericin and the identified biodegradational products support our conclusion (Figure 6).

On the basis of the results obtained, we believe that degradation of hypericin by the investigated fungi proceeds according to the mechanism shown in Figure 7.

The bactericidal effect of hypericin (10 mg/mL and 20 mg/mL) on some pathogenic bacteria was determined by identification of growth inhibition zones for 48 hour old cultures, applying the disc method with filter paper. The inhibition zones were graded according to the percentage of bacterial growth decrease and the results obtained are presented in Table 2.

Applied in the test concentrations, hypericin inhibited the growth of all investigated cultures. The cultures most sensitive to 10 mg/mL hypericin were E. coli and Enterococcus, while the cultures most sensitive to 20 mg/mL hypericin were Streptococcus A and B, and P. mirabilis.

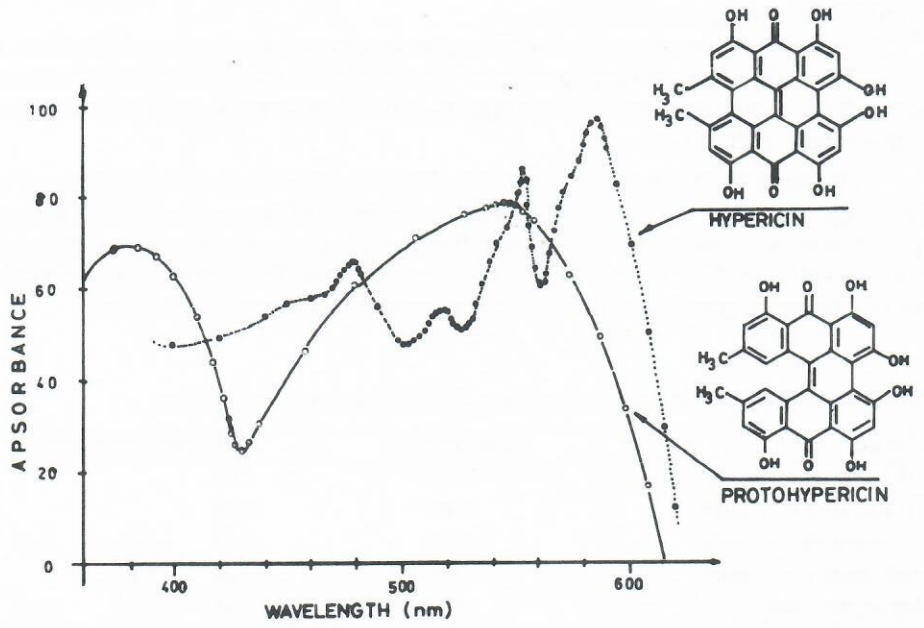

Figure 6. UV absorption spectra for hypericin and the biodegradational product protohypericin.

It may be concluded that, added in the concentration of 30 mg/mL to the standard nutrient base, hypericin influenced the biochemical reactions of fungi in two ways: Trichoderma, Penicillium and Doratomyces, were moderately inhibited, but vital and able to adapt to the changed conditions. Inhibition of Aspergillus development was several times higher, particularly during the first few days.

Metabolism of proteins, followed indirectly via the activity of proteolytic enzymes, was reflected in the decrease of the total activity.The maximal slowdown was registered with T.harzianum, somewhat less with P. verucosum and only negligibly with T. viride and D. stemonitis Maximal activity with T. viride was shifred from the 5th to the 7th day; but with D. stemonitis, this maximum was achieved on the 6th day instead of on the 9th, as expected. With A.niger PE was two times higher, without a time change.

Trichoderma, Penicillium, Doratomyces and Aspergillus genera involved hypericin in their metabolic reactions, slowly degrading it via protohypericin. Hypericin in the concentrations of 10 mg/mL and 20 mg/mL was bactericidal to Enterococcus, E.coli, Streptococcus, A and B, and P. mirabilis.

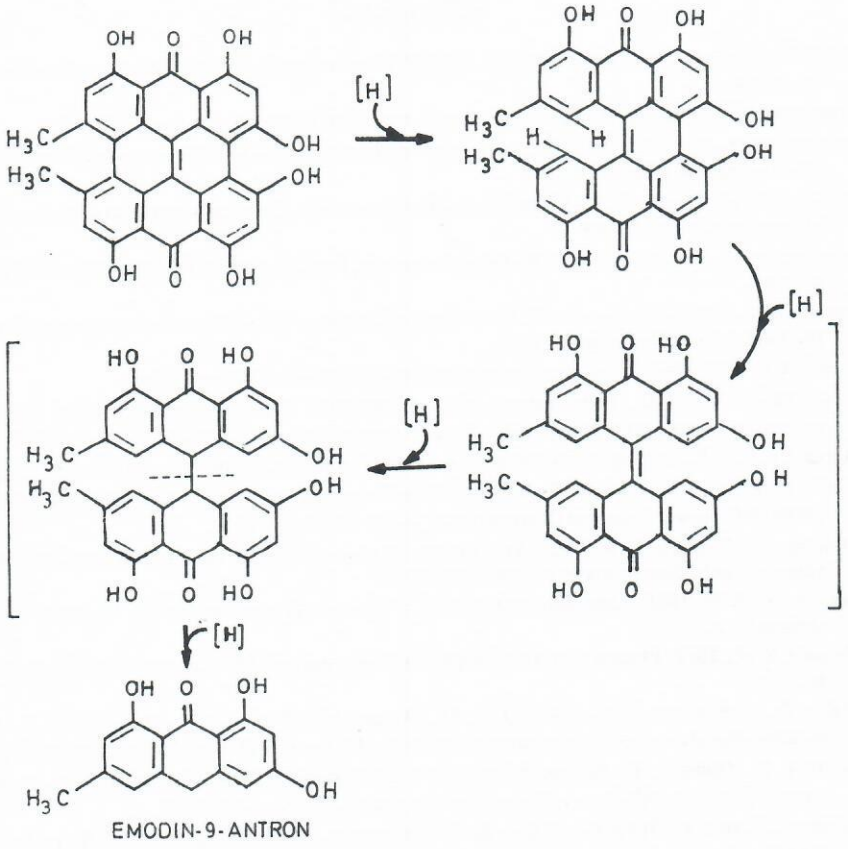

Figure 7. The proposed mechanism of hypericin biodegradation by the investigated fungi

Table 2. Bactericidal effects of Hypericin

Test organism	Inhibition level	
	10 mg/mL hyp.	20 mg/mL hyp.
Streptococcus B	+	+++
Streptococcus A	+	+++
Staphylococcus epidermides		+
Staphylococcus aureus	+	+
Enterococcus		+++
Pseudomonas aeroginosa		+
E. coli		+++
Klebsiellae		+
C. albicanis		+
P. mirabilis		+++

Key + denotes 10% inhibition; ++ denotes 25% inhibition; +++ denotes 50% inhibition

REFERENCES

1. *Bell G. R., Sadowski A. J. Matsckiner, J. T.* 1972. Mechanism of Action of Warfarin. *Biochemistry, 11*, 1959
2. *Birkinshaw, J. H. Gourlay, R.,* 1961. The structure of Democybin. *Biochem, J., 80,* 387
3. *Brockmann, H., Eggers. H.* 1958. Partiasyntese von Prot-Hypercin und Hypericin aus Penicillopsin, *Chemische. Berchte, 91*, 81-91.
4. *Brockmann, H., Neeff, R.* 1951. Oxidation entsteht deraus Oxypenicillopsin aus diesem durch Hypericin. *Naturw, 38*, 47.
5. *Brockmann, H., Eggers, H.* 1953. Partialsynthese von Proto-Hypericin aus Emodinatron. *Naturw, 40*, 461.
6. *Czekalowski, J. W. S., Karrzynski, B,* 1948. The breakdown of phenols and related compounds by bacteria *J. Gen. Microbiol., 2.* 231.
7. *Dudka, A. I.* 1982. Methods of experimental ecology (in Russian), *Nauka, Kiev.*
8. *Fukumoto, B.,Tsuru, D., Yamamoto, T.,*1969. Purification, crystallization and some enzymatic properties of acid proteases of Phrizopus chinensis, Bull. *Agr. Biol. Chem, 31.* 710-717.
9. *Happold, F. C.* 1950. Biological Oxidation of Aromatic Rings, *University Press Cambridge, England.*
10. *Koch, O. W., Kruger H. J.,* 1995. A Highly Reactive and Catalyticlly Active Model System for I Intradiol-Cleaving Catechol Dioxygenases, 34, N. 23/24, 2671.
11. *Kramer, N. Doetsch, R. H.* 1950. The growth of phenol-Ultizing bacteria on aromatic carbon sources. *Arch. Biochem and Biophys. 26,* 401.
12. *Naumova, R. P.* 1985. Microbial metabolism of no-natural compounds *(in Russian) Kazans. University, p.* 59-94.
13. *Neujahr, Y. H.,* 1972. Phenol degrading enzymes from yeast. IV IFS: *Ferment Technology Today,* 321-326.
14. *Roller, D. S., Bennett, H. P., Delaney, G. M., Mason, J. R. Stirling J. L., Thurston, C. F.,* 1984. Electron-transfer coupling in microbial fuel cells, *J. Chem. Techn. Biotechnology, 3413,3-12.*
15. *Shibata, S., Ogikaro, Y., Kabayashi, V.,Seo, S., Kitagawa, I* 1968. The revised structures of Luteoskyrin, Rubroskyrin and Rugulosin, *Tetrahedron Letters, 27*, 3179.
16. *Solujic, S. Comic, Lj. Sukdolak, S. Vucetic J., Stojanovic, J.* 1994. Microbiological degradation of the sesquiterpene type of synthetic lactone *(in Serbian) Mikrobiologija, Vol. 31. No 1*, 25-34.
17. *Sponer, D. F. Sybes, G.* 1972. Laboratory assessment of antibacterial activity. Methods in Microbiology, Acad. Press. London, 1972.
18. *Yoshido, F.,* 1956. Studies on the proteolytic enzyme of blac Aspergilli Part III The specificitly of crystalline aspergillus saibi proteinase on synthetic substrates. *Bull. Agr. Biol. Chem., 20. p.* 262-266.

BIOHEMIJSKE REAKCIJE NEKIH GLJIVA U PRISUSTVU HIPERICINA ISPITIVANE POD USLOVIMA IN VITRO

SLAVICA SOLUJIĆ, S. SUKDOLAK, LJILJANA ČOMIĆ, i LJ. KRSTIĆ

SADRŽAJ

U radu su dati rezultati istraživanja uticaja određene koncentracije policikličnog fenola hipericina (30 mg/mL) unetog u mikrobiološku podlogu na

biohemijske reakcije gljiva *A. niger, P. verrucosum, T. viride, T. harzianum and D. stemonitis.*

Hipericin se ponaša i kao aktivator i kao inhibitor biohemijskih reakcija zavisno od karakteristika gljiva. Sposobnost biodegradacije policikličnog fenola pod uticajem gljiva ispitivana je u hranljivoj podlozi posle 14 dana razvoja. Obim biodegradacije i broj biodegradacionih proizvoda je različit i uslovljen fiziološkim karakteristikama gljiva.

Ispitivani policiklični fenol baktericidan je u koncentraciji 10 mg/mL i 20 mg/mL prema *Streptococcus A. Streptococcus B, Enteroccocus, E. coli* and *P. mirabilis.*