Effects of Three Hypericum Species on Broiler Erythrocyte Antioxidant Enzyme Activities in Nitric Oxide Exposure

ÜÇ HYPERICUM TÜRÜNÜN NİTRİK OKSİTE MARUZ BIRAKILMIŞ CİVCİV ERİTROSİTİNE AİT ANTİOKSİDAN ENZİM AKTİVİTELERİ ÜZERİNE ETKİSİ

Sibel KONYALIOĞLU*, Gözde (ELGİN) MERAL** , Emrah KILINÇ***

* Department of Biochemistry, Ege University Faculty of Pharmacy,
** Department of Pharmaceutical Botany, Ege University Faculty of Pharmacy,
***Department of Analytical Chemistry, Ege University Faculty of Pharmacy, İzmir, TURKEY

Summary

Purpose: The aim of the present study was to investigate the probable antioxidant effect of the total extracts of *Hypericum perforatum* L., *H. empetiifolium* Willd., *H. triquetrifolium* Turra.

Materials and Methods: *Hypericum* extracts were incubated with broiler erythrocytes samples at room temperature. Thereafter catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities of broiler erythrocytes were determined spectrophotometrically. Measurements were performed in control, nitric oxide (NO) and three extract groups.

Results: During antioxidant capacity determinations in comparison to the control group, all antioxidant enzyme activities in NO group were significantly found to be lower ($p<0.05$). In the extract groups of each plant, in comparison with the NO group, significant increases were found ($p<0.05$); also in comparison with control groups insignificant differences were found ($p>0.05$).

Conclusion: Our results significantly ($p<0.05$) demonstrate that the other two species of *Hypericum* also have the same effect as *Hypericum perforatum,* which is known to exhibit antioxidant effect.

Key Words: Antioxidant enzymes; Nitric oxide, Hypericum perforatum, Hypericum triquetrifolium, Hypericum empetrifolium

T Klin J Med Res 2002, 20:32-36

Özet

Amaç: Bu çalışmanın amacı *Hypericum perforatum* L., *H. empetrifolium* Willd., *H. triquetrifolium* Turra.'nın total ekstrelerine ait olası antioksidan etkilerin incelenmesidir.

Materyal ve Metod: *Hypericum* ekstreleri oda sıcaklığında civciv eritrosit örnekleri ile inkübe edildi. Takiben civciv eritrositlerine ait katalaz (CAT), glutatyon peroksidaz (GPx) ve süperoksit dismütaz (SOD) aktiviteleri spektrofotometrik olarak belirlendi. Ölçümler kontrol, nitrik oksit (NO) ve ekstre gruplarında yapıldı.

Bulgular: Antioksidan kapasitenin belirlenmesi esnasında NO grubundaki tüm antioksidan enzim aktiviteleri kontrol grubu ile mukayese edildiğinde daha düşük bulunmuştur ($p<0.05$). Her bitkiye ait ekstre gruplarında, NO grubuna göre anlamlı artışlar ($p<0.05$), aynı zamanda kontrol gruplarına göre anlamlı olmayan farklılıklar ($p>0.05$) bulunmuştur.

Sonuç: Sonuçlarımız anlamlı biçimde ($p<0.05$), antioksidan etkisi daha önceden bilinen *Hypericum perforatum* gibi diğer iki *Hypericum* türünün de ayni etkiye sahip olduğunu göstermiştir.

Anahtar Kelimeler : Antioksidan enzimler, Nitrik Oksit, Hypericum perforatum, Hypericum triquetrifolium, Hypericum empetrifolium

T Klin Araştırma 2002,20:32-36

A free radical can be defined as a species capable of existing independently and containing one or more unpaired electrons (1). Oxygen free radical species (ROS) are highly reactive and potentially damage transient chemical species formed in all cells, as unwanted metabolic by-products of normal aerobic metabolism (2). Furchgott and Zawadski (3), for the first time identified the endothelium-derived relaxing factor, later identified as nitric oxide (NO) (4,5). NO, as a member of ROS, plays an important role in many physiological and pathophysiological processes (6,7). Many efforts are being made to understand the mechanism of how this gas molecule is

synthesized and reacts within various cells and biological systems.

Neurodegenerative diseases, aging, rheumatoid arthritis, metabolic diseases like diabetes, hypertension, etc. are considered to be the free radical mediated diseases. Cells are protected from ROS induced damage by a variety of endogeneous ROS scavenging enzymes (eg. SOD, GPx and CAT) and chemical compounds (eg. vitamins E, C and glutathione) known as antioxidants. SOD catalyses the dismutation reaction between two superoxide radicals (0_2"), yielding hydrogen peroxide and molecular oxygen. The detoxification of hydrogen peroxide is then catalysed by GPx or CAT.

In the last decade, a worldwide trend towards the use of natural phytochemicals present in various herbal products with antioxidant activity have been proposed for use in functional foods (8). Natural antioxidants may have applications in the food industry and there are some evidences that these substances may have an antioxidant effect within the human body after consumption (9).

Hypericum species, have been ethnomedically used in different parts of Turkey for their many biological effects such as antiseptic, sedative, antihelmentic, wound healing, antiviral, antibacterial and hepatoprotective (10-12). In our previous studies, we demonstrated the relaxing effect of methanolic extract of *H. triquetrifolium* on smooth muscles and its antinociceptive activity for the first time (13, 14). Herbal medicine of *Hypericum* species (esp. H. *perforatum)* have also been used as antidepressant in clinic use in USA and other European countries (15). Yet such effects of some *Hypericum* species have not studied in the last decade thus the purpose of this work was to compare the antioxidant activity of alcoholic extracts of three *Hypericum* species by antioxidant enzymatic defence determinations.

Materials and Methods

Chemicals: RANDOX-Ransel and RANDOX-Ransod enzyme kits were used for GPx and SOD assays, respectively. Chemicals used were as follows; methanol (Lab-Scan C2517), hydrogen peroxide, sodium nitrite, potassium dihydrogen phosphate and disodium hydrogen phosphate (Merck). Other chemicals were of analytical grade and used without further purification. All aqueous solutions were prepared with bidistilled water.

Plant Material: Fresh plants of *H. perforatum* from wild collections of KaragoT, Izmir June 1999, *H. triquetrifolium* of Karaali village, Manisa October 1999 and *H empetrifolium* from Urla, Izmir July 1999 were used. Mainly, the aerial parts of the plants carrying a high proportion of buds and flowers were selected. The plant was identified by em. Professor E. Sauer, Saarland University, Institute of Botany, Germany. Voucher specimens of the plants were kept for record in the herbarium of Ege University, Faculty of Pharmacy, Department of Pharmaceutical Botany (Voucher no. 5434, 5435, 5436 resp.).

Extraction of Hypericum species: The crude drug was dried in shade and finely powdered by a mill (Brabender OHG, Duisburg). A modified method of Wagner and Bladt was used for the extraction of the powdered plant (16). Methanol at 80 °C was used for soxhlation (750 mL methanol/100 g crude drug) and the extracts were dried in vacuo (yields were 25.94 , 36.2 and 26.36, resp.). After lyophilization (Labconco lyophilizateur, - 50 °C) of the extracts (yields were 87.48, 76.32 and 80.67 % resp.) they were dissolved in a mixture of Tween 20 and bidistilled water (1:9) and were administered to erythrocyte hemolysates.

Preparation of NO solutions: NO gas was produced under N_2 atmosphere in a tightly closed glass gas producing chamber, where 125 mL of 6M H_2S0_4 was added in portions on to 50 g $NaN0_2$. Simultaneously produced NO gas was delivered through connected tubes to a 100 mL solution in wash bottle, where the NO saturated solution was obtained by bubbling NO gas through deoxygenated bidistilled water for 30 min, reaching to a value of ~2 mM (1.9 mmol.L"') concentration at saturation (17). A series of standart NO solutions were prepared by diluting the NO saturated solution and were used in NO exposure of the enzymes.

Preparation of blood samples: One day old broiler chicks of commercial strain, Hubbard, were obtained and fed as described previously (18). A sample of 2 mL of blood collected from the wing veins of fifteen 35 day old chicks was immediately placed in a heparinised tube. Whole heparinized blood from broilers was used for GPx activity and Hb determination. Hb concentrations were measured by using Drabkin Method. Erythrocyte hemolysate was used for SOD and catalase activity. Fresh blood samples from broilers were centrifuged at 3000 rpm for 10 min, plasma and buffycoat were removed. Erythrocytes were washed with %0.9 NaCl solution three times and erythrocytes were hemolyzed with ice-cold distilled water. The erythrocyte hemolysate was diluted with phosphate buffer (O.Olmmol.L^{-1}, pH: 7.0). Hemolysate of erythrocytes obtained from 15 chicks was divided into 3 as; control group, NO group and the extract groups.

Control Group: 0.5 mL of erythrocyte hemolyzate was incubated with 2 mL phosphate buffer (O.Olmmol.L^{-1}, pH: 7.0).

NO Group: 0.5 mL of erythrocyte hemolyzate was treated with lppm NO in 2 mL phosphate buffer (O.Olmmol.L^{-1}, pH: 7.0) for 5 minutes at room temperature.

Extract Groups: 0.5 mL of erythrocyte hemolyzate was treated with lppm NO and each of *Hypericum* extract solution (500 ug.mL^{-1}) in 2 mL phosphate buffer (O.Olmmol.L^{-1}, pH: 7.0) for 5 minutes at room temperature and enzyme activity was determined immediately.

Methods

Catalase activity was determined using the method of Aebi (19). The decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm. The results were expressed asK/gHb.

SOD activity was determined using the RANDOX-Ransod enzyme kit. This method employs xanthine and xanthine oxidase (XOD) generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium-chloride to form the red formazon dye. The superoxide dismutase activity was then measured by the degree of inhibition of this reaction. The results were expressed as U/g Hb.

GPx activity was determined using the RANDOX-Ransel enzyme kit. This method is based on that of Paglia and Valentine (20). in this method, GPx catalyses the oxidation of glutathione (GSH) by hydrogenperoxide. In the presence of glutathione reductase (GR) and NADPH (reduced nicotin amide adenin diphoshate), the oxidised glutathione (GSSG) was immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$ (oxide form). The decrease in absorbances at 340 nm were mesured. The results were expressed as U/g Hb.

Statistical Evaluation

The results given here are the mean±SD of fifteen separate experiments. The level of significance has been evaluated by ANOVA Oneway and Tukey HSD istatistical analysis methods using SPSS for Windows version 6.0 (SPSS, USA).

Results and Discussion

During antioxidant capacity determinations in comparison with the control group, all antioxidant enzyme activities in NO group were found to be significantly lower ($p < 0.05$). In the extract groups of each plant, in comparison with the NO group, significant increases were found ($p < 0.05$); also in comparison with control groups insignificant differences were found ($p > 0.05$). The experimental data on the activities of GPx, SOD and CAT are displayed at Table 1.

Antioxidant enzyme capacity of erythrocytes can be a suitable model since erythrocytes can be easily obtained, homogenized and stored. Due to these properties, antioxidant enzyme capacity determination was chosen.

NO, with its increasing popularity, was found to be responsible for various physiological and pathophysiological processes in the recent years. The inactivating effect of NO on various physiologically important enzymes such as ADH,

fable 1. Erythrocyte GPx, SOD, CAT activities of control, NO and Hypericum perforatum (HP), H. triquetrifolrurn (HT) and H. empetrifolium (HE) extract groups (± SD, n=15)

JSnzyme	Control Group	NO Group	HP Extract	HT Extract	HE Extract
GPx(UVgHb)	17.26 ±2.36	5.50±2.22*	14.75 ±2.22"	12.50 ± 2.50"	12 45 ±4 69"
SOD(U/gHb)	38.42 ±6.07	12.32 ±4.78*	29.48 ±2.36"	24.57 ±3 22"	24 78 ±5 21"
^CAT (K/gjjb)	376.98 ± 32.88	33.65 ± 7.59'	346.02 ± 26.91"	351.43 ± 30.42"	354.73± 34.08"

(): p<0.05, C)-P>0.05 (in comparison with control group, according to ANOVA Oneway and Tuckey HSD statistical analysis)

CAT, NADPH oxidase, GPx and SOD has previously been shown (21). Thus, in antioxidant enzyme capacity determinations, NO was chosen as the free oxygen radical.

In the control group of our work, antioxidant enzyme activity of broiler erythrocytes has significantly decreased as was expected in NO oxidative stress model. However, in the extract groups with the use of these three *Hypericum* extracts, the deactivation of the antioxidant enzymes were observed to be lower in the oxidative stress provided by NO.

As a result, the most popular *Hypericum* species in the world today is *H. perforatum,* therefore our aim was to compare antioxidant capacity of two other *Hypericum* species widely available in our region with H. perforatum. Our results showed that these *Hypericum* species also have antioxidant effect.

It has initially been discovered that compounds such as flavons, phenols, glycosides and terpenoids were responsible from the antioxidant effect of *H. perforatum* (15). Phenols, flavanoids, alkaloids, terpenoids, glycosides, organic acids and lipids act as natural free radical scavengers (21). In order to identify the origins of the antioxidant effects of these two *Hypericum* species, additional HPLC and GC-MS studies are planned. Many more species of the *Hypericum* genus are present in Turkey on which similar antioxidant studies are planned.

It is also possible that *Hypericum* species extracts may influence other cellular systems more detailed examinations of further antioxidant parameters are required.

REFERENCES

1. Greenwood, NN. Chemistry of the Elements. In: Earnshaw A, ed. Oxford: Pergamon press, 1990: 713-8.
2. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. Methods inEnzimol 1990; 186: 1-85.
3. Furchgott RS, Zawadski JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288: 373-6.
4. Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987; 327: 524-6.
5. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sei USA 1987; 84 : 9265-9.
6. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J 1994; 298 : 249-58.
7. Nathan CF. Nitric oxide as a secretory product of mammalian cells. FASEB J 1992; 6: 3051-64.
8. Fair DR.: Functional foods. Cancer Let 1997; 114: 59-63.
9. Cook MC, Samman S.: Flavanoids-chemistry, metabolism, cardiovascular effects and dietary sources. Nutritional Biochem 1996; 7: 66-76.
10. Mabey E, Mc Intyre M, Michael P, Duff G, Stevens J. The New Age Herbalist. New York: Macmillian Publishing Company, 1988: 61.
11. Kitanov GM. Hypericins in Hypericum Species. J Pharm Univ Mar 1995; 11(1-2): 343-50.
12. Ozturk Y, Aydin S, Baser KHC, Kirimer N, Kurtar-Ozturk N. Hepatoprotective Activity of Hypericum perforatum L. Alcoholic Extract in Rodents. Phytotherapy Res 1992; 6: 44-6.
13. Apaydin S, Goldeli E, Karamenderes C, Meral G, Zeybek U, Tuglular I. The Inhibitory Effect of Hypericum triquetrifolium Turra. Extract on Rat Aortic Smooth Muscle Contraction. Turkiye Klinikleri J Medical Res 2001; 19 : 24-30.
14. Apaydin S, Zeybek U, Ince I, Elgin G, Karamenderes C, Ozturk B, Tuglular I. Hypericum triquetrifolium Turra. extract exhibits antinociceptive activity in the mouse. J Ethnopharm 1999; 67 : 307-12.
15. Tripathi YB, Pandey E. Role of alcoholic extracs of shoot of Hypericum perforatum Linn on lipid peroxidation and various species of free radicals in rats. Ind J Exp Biol 1999; 37: 567-71.

16. Wagner I, Bladt S. Pharmaceutical quality of hypericum extracts. Journal of Geriatric Psychiatry and Neurology, 1994; 7(1): 65-8.
17. Mesaros S, Grunfeld S, Mesarosova A, Bustin D, Malinski T. Determination of nitric oxide saturated (stock) solution by chronoamperometry on a porphyrine microelectrode. Anal Chim Acta 1997; 339 : 265-70.
18. Altan O, Altan A, Oguz I, Pabuccuoglu A, Konyalioglu S. Effects of heat stress on growth, some blood variables and lipid oxidation in broilers exposed to high temperature at an early age. British Poultry Science 2000; 41: 489-93.
19. Aebi A. Catalase in vitro. Methods in Enzymology 1984; 105: 120-7.
20. Paglia DE, Valentine WN. Studies on the quantitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70: 158-9.
21. Feher J, Cosmos G, Vereekei A. Free radical reactions in medicine. Suttutgart: Springer-Verlag, 1992: 1-42.

Received: Oct. 25,2001

Correspondence: Sibel KONYALIOGLU
Ege University Faculty of Pharmacy
Department of Biochemistry,
Bornova 35100 IZMIR-TURKEY
konyalioglus@pharm.ege.edu.tr