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Artículo Científico

Antioxidant and scavenging activity of skyrin on free radical and some reactive oxygen species.

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Resumen:

El skyrin es un producto natural proveniente de algunas especies botánicas (hongos) y es uno de los primeros agentes antidiabéticos no-peptídico de pequeño peso molecular. El objetivo de este estudio fue el de investigar la habilidad del skyrin en inhibir radicales libres y algunas especie oxigenadas reactivas como $^{\cdot}OH$, $^1O_2$, H_2O_2 generados en sistemas libres de células usando la quimioluminiscencia del isoluminol, luminol y espectroscopía de absorción como monitores. En los ensayos de quimioluminescencia del isoluminol (ILCE) en presencia de peroxidasa de rábano (HRP) y en los del luminol – H2O2 o ión férreo, fue observado una inhibición dependiente de la cantidad de skyrin adicionada a este medio. Por otro parte, el skyrin mostró una actividad secuestradora del galvanoxyl radical en soluciones etanólicas. En un experimento separado el skyrin fue capaz de atrapar oxígeno singlete ($^1O_2$) generado mediante la irradiación con luz visible de rosa de bengala. La actividad antioxidante del skyrin se comparó con otros antioxidantes conocidos como la emodina, dipiridamol y las vitaminas C y E en diferentes ensayos como también en peroxidación lipídica. Estos resultados sugieren que el skyrin atrapa especies reactivas de oxígeno y de radicales libres en grado similar que la emodina, vitamina C y E o al del dipiridamol. **Palabras Clave:** Quimioluminiscencia; skyrin; emodin; atrapador de radicales; actividad antioxidante.

Abstract

Skyrin is a natural product from some botanical species (fungi) and the first small molecular weight nonpeptidic antidiabetic agents. Now, the objective of this study was to investigate the ability of skyrin to inhibit free radical and some reactive oxygen species as $^{\cdot}OH$, $^1O_2$, H_2O_2 generated in cell-free systems using isoluminol and luminol-enhanced chemiluminescence and electronic absorption spectra as monitors. In the presence of skyrin a dose-dependent inhibition period was observed in this system as assayed by isoluminol-enhanced chemiluminescence (ILCL) in the presence of horseradish peroxidase (HRP), as well as by luminol-enhanced chemiluminescence (LCL) in the presence of H_2O_2 or ferrous ion. On the other hand, skyrin showed an efficient scavenging activity of galvanoxyl radical in ethanolic solutions. In a separate experiment the trapping of singlet oxygen ($^1O_2$) generated by rose bengal in the presence of skyrin was observed. The antioxidant activity of skyrin was compared with that of known antioxidants as emodin, dipyridamole, vitamins C and E in different assays as also in the lipid peroxidation. These results suggest that skyrin scavenge reactive oxygen and free radicals species in a comparative grade that emodin, vitamin C and E or dipyridamole. **Keywords:** Chemiluminescence; skyrin; emodin; free radical scavenger; antioxidant activity.

Introduction

At the present time, natural products from some botanical species have become the most important source of skyrin which is of potential biological interest as scavenger of free radicals and reactive oxygen species. The existence in the American continent of an immense diversity of these natural products from a variety of species, especially in countries located in the neo-tropical region (Venezuela among other) and the fact that a great percentage of them has not been subjected to scientific investigation, open a great opportunity for new areas of research in Chemistry and Ecology[1, 2].

The species *Rhizophora* mangrove, popularly known as "Red Mangrove" and commonly found in the neo-tropical

regions of the American continent, has been a plant of special interest in these investigations because as scientists tried to find out the most frequent cause of mortality of these trees, they discovered four types of fungi as the possible pathogenic agents responsible for their death, in some cases of the whole adult plant and in others of the progressive death of the different parts of the plant such as branches and roots. *Cytospora* was reported for the first time by Kohlmeyer as *Cytospora rhizophorae* in 1969, more recently it has been reported that several interesting compounds of potential biological interest have been isolated after air fermentation of cultures of this fungus[3-10].

One of the first works reported was that of Gurusiddaiah and Ronald who isolated three macrocyclic compounds that showed antibiotic activity and which called Grahamimycin A, A1 and B[11]. Hanson and collaborators isolated and identified in addition to the former two new macrocyclic compounds that in contrast to those isolated by Gurusiddaiah and Ronald, were identified as 13 member ring compounds called Bartanol and Bartallol[12]. Brady and collaborators carried out assays of antibiotic activity of different organic extracts from cultures of *Cytospora sp* isolated from the plant *Conocarpus erecta*. From the most active extract five new compounds were isolated which were called *Cytosporona* A, B, C, D and E, two of them were identified as two bis-anthraquinone isomeric compounds denominated as Cytoskyrin A and B respectively[13]. The structures of these compounds are shown in the following figure.

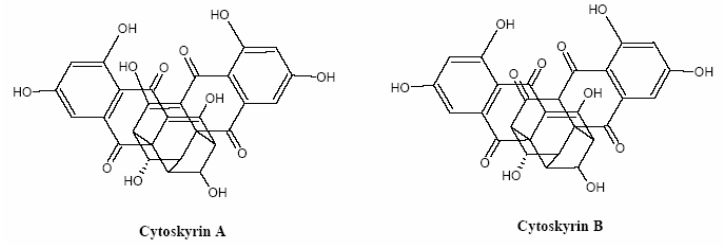


Figure 1. Structures of the cytoskyrin A and B.

These authors carried out assays of biochemical induction, a quick bacterial colorimetric test to identify compounds that damage or inhibit the synthesis of DNA, making possible the identification of a natural product with potential anticancer activity. In this sense the Cytoskyrin A demonstrated to possess high activity while the Cytoskyrin B didn't present any detectable activity.

Skyrin (figure 2) was discovered by Howard and Raistrick in 1954. It was isolated from Penicillum islandicum Sopp. These authors studied their chemical behaviour proposing a structure starting from the obtained results[14]; then Shibata and co-workers characterized it by using spectroscopic methods (UV, IR, NMR), corroborating the structure proposed by Howard and Raistrick. Hatfield and Slagle in 1973 were able to isolate and to characterize skyrin of extracts of *Hypomyces lactifluorum*, and they also reported spectral data, UV, IR and NMR for this compound[15-18].

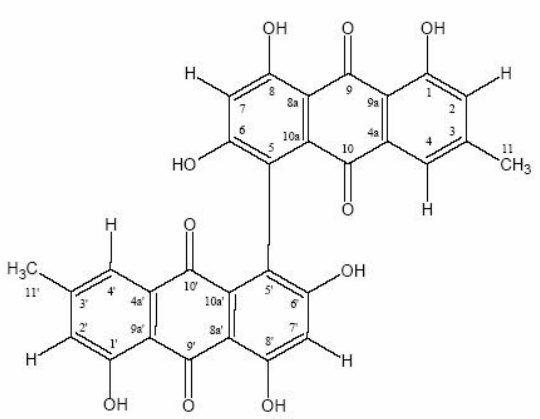
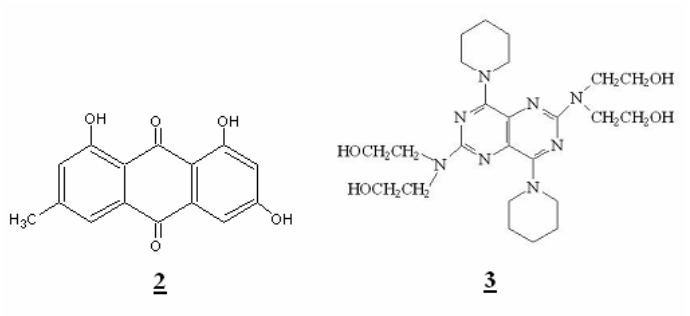


Figure 2. Structures of the hydroxyanthraquinone skyrin (**1**).

Skyrin (**1**) is the first small molecular weight nonpeptidic agent demonstrated to interfere with the coupling of glucagon to adenylate cyclase independently of binding to the glucagons receptor. The effective consequence of this is that skyrin specifically blocks the metabolic sequelae of glucagon action in hepatocytes[19]. Whereas the modest potency of skyrin limits its own therapeutic usefulness, it is likely to be a useful tool to explore the potential of a mechanistically novel class of antidiabetic agents.

As far as the research carried out in this laboratory all the information about the events at the molecular level was obtained via optical studies, such as electronic absorption spectra and chemiluminescence is of vital importance for the determination of the antioxidant and scavenging activity of this group of compounds on free radical and reactive oxygen species (ROS). The search of this information prompted us to carry out an extensive study using both techniques in homogeneous solvents on hydroxyl and galvanoxyl radicals[20-21], sensitized peroxidation of linoleic acid, as well on singlet oxygen[22-26]. The latter is chemically very reactive, inducing oxidation of proteins, lipid peroxidation and DNA damage. Moreover, $^1O_2$ mediates the induction of some signaling pathways by UVA, culminating in modulated gene expression. Singlet oxygen is an important intermediate in the damaging effects caused by UVA and in photodynamic therapy on cutaneous processes, such as apoptosis, among others. The combined approach proved to be useful in understanding the mechanisms by which these hydroxyanthraquinones develop their antioxidant activities.



2 **3**

Figure 3. Structures of the antioxidants emodin (**2**) and dipyridamole (**3**).

2. Materials and methods

2.1. Chemicals

Skyrin (**1**) was obtained from extraction with ethyl acetate/methanol (1:1) of the fungus *Cytospora rhizophorae* of the *Rhizophora* mangrove. The soluble fraction in organic solvents contains as majority compound Skyrin (18%). The purification was carried out by TLC (0.2 mm, silica gel $60F_{254}$ (Merck, Darmstadt, Germany) and by liquid chromatography (HPLC, Waters Delta Prep 4000) equipped with an analytic and preparative C18 Phenomenex (250 x 4.60 mm) column using a CH_3CN/H_2O gradient as mobile phase at a flow rate of 1.0 $ml.min^{-1}$, with UV-Vis (PDA) monitoring. The isolated products were analyzed by ^{1}H-NMR spectroscopy (Bruker Aspect 3000, 300 MHz and 500 MHz), FT IR (Nicolet DX V 5.07), UV-Vis absorption spectrophotometry using a Milton-Roy Spectronic 3000 array instrument (Milton Roy Company-USA) and Lambda650 spectrophotometer, Perkin Elmer and GC-MS (Carlo Erba/Kratos MS25RFA).

Skyrin : Orange crystals from acetone/pyridine 10:1, m.p. >300ºC. UV (MeOH) λmax 210, 257, 300, 461 nm (log ε : 5.12, 4.75, 4.53, 4.40); IR (KBr) 3420, 1625, 1600, cm^{-1}; ^{1}H-NMR (DMSO-*d6*, 300,13 MHz) δ 2,30 (6H, s, H-11); 6,59 (2H, s, H-7,7'), 7,10 (2H, br s, H-2,2') 7,24 (2H, br s, H-4,4'), 12,13 (br s, OH), 12,80 (br s, OH-8,8'); ppm. (see Fig. 2). ^{13}C-NMR (DMSO-*d6*, 300,13 MHz) δ 189,50 (C-9,9'), 182,10 (C-10,10'), 165,06 (C-6,6'), 164,40 (C-8,8'), 161,02 (C-1,1'), 148,02 (C- 3,3'), 133,26 (C-4a, 4a'), 131,24 (C-10a,10a'), 124,06 (C-5,5'), 123,48 (C-4,4'), 120,37 (C-2,2'), 113,08 (C-9a,9a'), 108,66 (C-8a,8a'), 107,11 (C-7,7'), 21,50 (C-11,11'). Assayed with DEPT-NMR (θ =135º) and 2D-NMR (COSY, HMQC and HMBC). This characterization was compared with the previously published data, turning out that the analytical data was very close to that of Ogihara, Takeda, and Hatfield within experimental error[17-19].

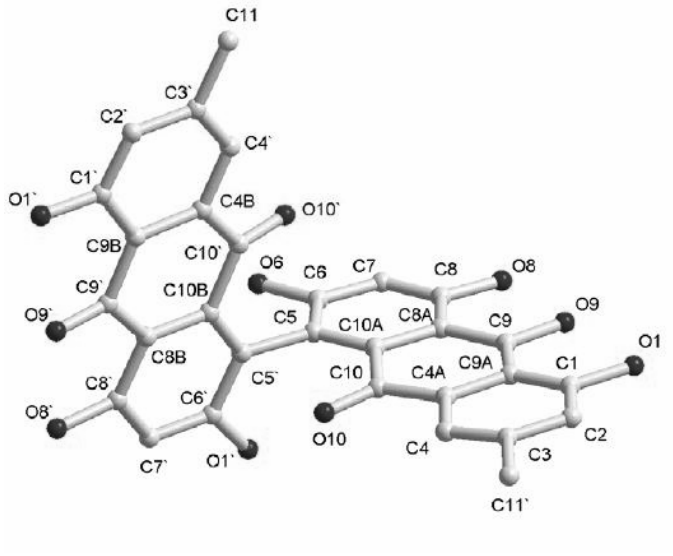


Figure 4. 3D-structure of skyrin (**1**) with C-numeration.

Emodin, dipyridamole, galvanoxyl radical, horseradish peroxidase (HRP), tocopherol acetate and ascorbic acid were purchased from Sigma (St. Louis, MO, USA). Their purity was 99.2% as determined by ^{1}H NMR-spectroscopy (Bruker Aspect 3000, 300 MHz) and UV-Vis spectrometry (Milton-Roy Spectronic 3000 array and Lambda35 spectrophotometer, Perkin Elmer). 3-aminophthalhydrazide (Luminol), 4-aminophthalhydrazide (isoluminol), rose bengal and ferrous sulfate heptahydrate were purchased from Aldrich (Milwaukee, USA). All analytical or HPLC grade solvents were obtained from Merck (Darmstadt, Germany).

The relative quantum yields of fluorescence for skyrin were determined at room temperature either by comparing the corrected fluorescence intensity of the these complexes in ethanol-H_2O with that of rhodamine B (at a concentration of 1 x 10^{-7} M in ethanol; fluorescence quantum yield, 0.69) or with that of quinine bisulfate in 0.05 M H_2SO_4 (fluorescence quantum yield, 0.55)[27].

2.2. Chemiluminescence (CL) generated in cell-free systems; H_2O_2-induced CL

H_2O_2 (3.5 mM) was dispenser to phosphate buffered saline solution (PBS, 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4) and luminol (250 μM, prepared daily in 2 M NaOH and diluted with PBS) and the corresponding anti-oxidant (skyrin, emodin, vit. C, E and dipyridamole) at different concentrations or PBS used as a blank (0.5%). The generated CL at 37 ºC was measured continuously for 10 min in a Luminoskan Ascent luminometer (Thermo-Labsystems, Finland) in a 96-well Thermo Labsystems Microtiter plate[24,28,29].

2.3. CL generated in cell-free systems; Ferrous iron-induced CL

Hydroxyl radical was generated by addition of ferrous iron to the buffer solution as described previously [29]. Freshly prepared $FeSO_4$ (40 μM) was added to the PBS plus luminol (250 μM) mixture and CL was recorded continuously for 10 min at 37 ºC.

2.4. Isoluminol amplified chemiluminescence

Chemiluminescence was measured in a Luminoskan Ascent (ThermoLabsystems, Finland) using 96-well Thermo Labsystems Microtiter plates, containing 56 μM isoluminol, 4 U (HRP) and the corresponding anti-oxidant (skyrin, emodin, vit. C, E and dipyridamole) at different concentrations or PBS as a blank (0.5%). The emitted light was recorded as luminescent units at 20 s. intervals during 10 min at 37 ºC. All results were expressed as percentages of the control (areas under the luminescent units (rlu), relative light units vs. time curves).

2.5. Reactions with galvanoxyl radical

Titrations of equimolar ethanol solutions of galvanoxyl radical (5.0×10^{-5} M) were carried out with aliquots of 50 μL of the corresponding ethanolic solutions of skyrin. The course of the reaction was followed by UV-Vis spectrophotometry (for 10^{-7} M solutions at 435 nm) using a UV-Vis spectrometry (Lambda35 spectrophotometer, Perkin Elmer). The same process was carried out in presence of emodin, vit. C, E dipyridamol (DIP), and compared with the skyrin reaction.

2.6. Reactions with singlet oxygen

Photosensitized degradation of **1** was carried out using rose bengal in ethanol as $^1O_2$ sensitizer. The solutions were irradiated under aerobic conditions at room temperature, with an Osram HQL 250 W medium pressure Hg lamp located inside a pyrex immersion-well photoreactor (Applied Photophysics parts No. 3230 + 3307) for visible range irradiation (spectral output 400-600 nm) with a maximum at 420 nm with a total irradiance of 21 mW/cm^2 as measured with a model of UVX Digital Radiometer after 1 h continued illumination. The distance between the light source and the test aliquots was 10 cm. The temperatures detected in the cuvette during a standard 1 h irradiation were no higher than 27 °C. This process was monitored spectrophotometrically, as well as by gas chromatography and thin layer chromatography.

Indirectly, photosensitized degradation of histidine (a selective singlet oxygen scavenger) was measured in the presence of 1.5×10^{-5} M solution of rose bengal in ethanol. This solution was mixed with an equivalent quantity of L-histidine solution at 0.60 to 0.74 mM in phosphate buffer 0.01 M, pH 7.4. Samples of this mixture were irradiated at time intervals from 45 to 60 min. with the respective controls being protected from light. The concentration of histidine was determined by a colorimetric reaction using phosphate buffer, sulfanilic acid, sodium nitrite, sodium carbonate and ethanol as reagents. The optical density was read on a spectrophotometer at 440 nm against a blank reagent, a modified Pauly reaction and by bleaching of p-nitrosodimethylaniline[30,31]. The same experiment was carried out in presence of different concentrations (0.25, 0.50, 1.0, and 1.5×10^{-5} M) of skyrin and compared the competition of the scavenging process of the histidine.

2.7. Antioxidant activity of skyrin on sensitized peroxidation of linoleic acid

Linoleic acid 10^{-3} M in PBS was oxidized by using the method described by Yen and Chen[32] in the presence of skyrin, emodin, dipyridamol, vitamins C and E (1.0×10^{-4} M), and monitored by UV-spectrophotometry, reading the absorbance at λ = 500 nm on a Milton-Roy 3000 spectrophotometer after colouring it with $FeCl_3$ and thiocyanate at intervals during incubation at 37 ºC. The formation of dienic hydroperoxides was also monitored by UV-spectrophotometry, through the appearance and progressive increase of a new band at λ = 233 nm [33]. This test was also carried out under argon atmosphere[24].

2.8. Statistical treatment of results

At least three independent experiments were performed except where indicated otherwise. The results are expressed as a mean ± S.E.M. derived from 3-4 observations. The level of significance accepted was $p \leq 0.05$.

3. Results

All spectra were carried out at pH 7.4 to maintain equality in all the experiments. The absorption spectrum of the skyrin showed four principal band 218 nm (2.9), 258 nm (2.0), 298 nm (1.2) and 466 nm (0.8) with a molar extinction coefficient of $\varepsilon_{max} = 80.000 \pm 55$ at $\lambda_{max} = 258$ nm (fig. 4). Skyrin is relatively photostable under irradiating with UV-A and visible light. This property was carried out monitoring their absorption bands at 258 nm and 466 nm as a function of light absorbed during 5 hour.

The most important band of the emission spectra of skyrin is at 355 nm with an excitation at 304 nm. The relative quantum yields of fluorescence at pH 7.4 or in ethanol (Φ_F) = 0.031 was comparatively a little higher that its similar monomer aloe emodin 0.020.

The chemiluminescence (CL) observed both in the processes induced by H_2O_2 as well as by ferrous ion in luminol or else by HRP in isoluminol was used to evaluate the scavenging capacity of skyrin as compared to that of emodin, dipyridamole, α-tocopherol (vit. E) and vitamin C on ROS. Studies on the first two hydroxyanthraquinones one could observe a deceleration and quantitative decrease of the production of CL.

The CL activity generated by isoluminol and HRP reflects the release of reactive oxygen species (ROS) especially $^{\cdot}OH$[34]. Hydroxyl radical was also generated by the addition of a freshly prepared $FeSO_4$ solution to the mixture containing luminol and measured by chemiluminescence. These results are in agreement with the previous observation with other drugs[29] where the addition of a ferrous ion salt to buffered solutions generates the hydroxyl radical-mediated oxidative reactions. The scavenging activity of skyrin on ROS is shown in figure 5. In this assay system emodin was more effective than skyrin and at the same time the latter was better than dipyridamole as compared with standard antioxidants α-tocopherol and vitamin C.

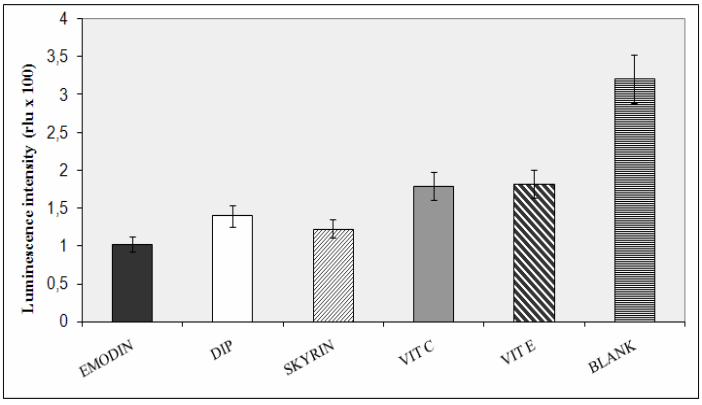


Figure 5. Quenching effect of the luminol chemiluminescence (H_2O_2 = blank) observed for emodin, dipyridamole (DIP), skyrin, vitamin C and E. Data in the inset are shown as mean ± SD (n = 3).

The reactivity of the skyrin toward galvanoxyl (a model phenoxyl radical) in ethanol solution was also investigated. Figure 6 shows the rate of this scavenging activity. A noticeable decrease in the absorption band at 435 nm was observed. This process can be of great analytic utility for the determination of the free radicals scavenging capacity of compounds with suspected antioxidant properties. The rates of the antioxidant activity (scavenging of galvanoxyl radical) of the skyrin was directly proportional to its concentration. In this assay emodin was more effective as scavenger than skyrin, and these showed bigger activity as anti-oxidants that the vitamins C and E and dipyridamole.

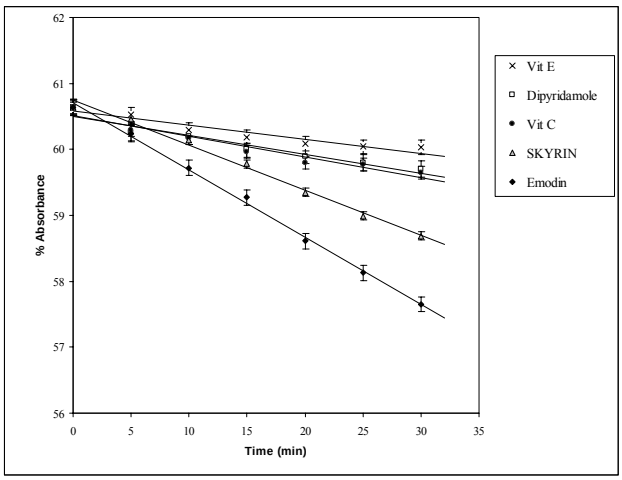


Figure 6. Comparative scavenging effect of the antioxidant under investigation [5.0 x 10^{-5} M] by monitoring decreasing of the absorbance of galvanoxyl radical at their wavelength maxima (435 nm) vs time. Data in the inset are shown as mean ± SD (n = 3).

The capacity of skyrin on singlet oxygen trapping (Figure 7) was also studied because the great participation in the oxidative activity and genotoxicity. In this sense, the photosensitized degradation of skyrin and the use of rose bengal in ethanol as $^1O_2$ photosensitizer were studied spectrophotometrically. The processes of photo-oxygenation by rose bengal have a quantum yield of singlet oxygen production > 0.6. We found that the generation of singlet oxygen, determined by the histidine assay, in presence of skyrin was lower than in its absence of this. Finally, singlet oxygen traps, compounds that react with $^1O_2$ to yield specific and more or less stable reaction products will be analyzed by HPLC in future investigations.

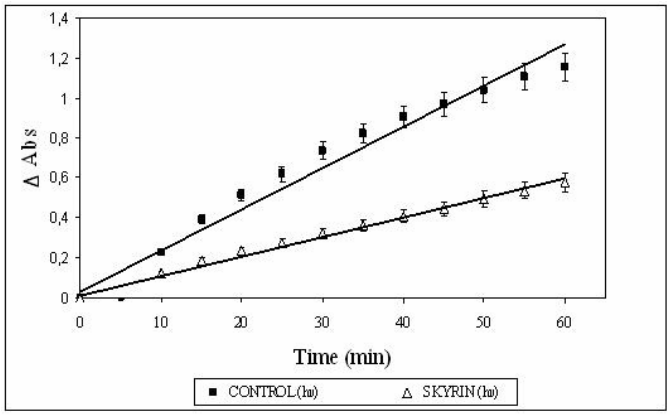


Figure 7. UV monitoring at 440 nm of the singlet oxygen quenching by addition of skyrin (control without skyrin) during the irradiation of rose bengal in the presence of histidine and p-nitrosoaniline. Data in the inset are shown as mean ± SD (n = 3).

The process of lipid peroxidation in the presence of skyrin was only circa 14% delayed; while in presence of the same concentration of emodin, vitamins E and C and dipyridamole the induced lipid peroxidation was inhibited about 16, 28, 43 and 57% respectively after 2 hour of peroxidation (Fig. 8).

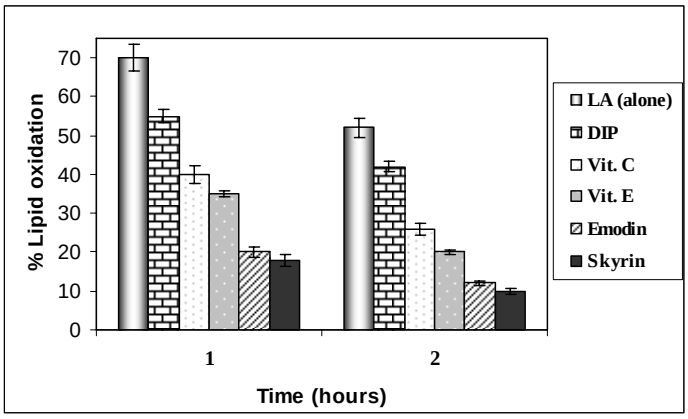


Figure 8. Antioxidant activity of emodin, dipyridamole (DIP), skyrin, vitamin C and E on lipid peroxidation of linoleic acid (LA). Data in the inset are shown as mean ± SD (n = 3).

4. Discussion

At the moment many nutritional supplements, depilatory creams, cleansing lotions, body lotions are prepared in combination of vitamins with stimulants or anti-oxidants of vegetable origin (plant derivatives). Some of them are: silymarin a flavonoid (green tea)[35,36], curcumin[37], ginger, resveratrol[38], dipyridamole, emodin, aloe-emodin and rhein[39,23-25]. Skyrin as well as emodin, as was evidenced in this work, are potent scavengers for radicals and specially ROS generated by cell-free systems, which for example could be generated in the skin by UV irradiation[40,41]. The experiments carried out in our laboratory have shown that skyrin acting as a hydroxyl radical scavenger as was detected by the chemiluminescence assays. In this test emodin and skyrin had similar anti-oxidant activity and at the same time the latter was better than dipyridamole, α-tocopherol and vitamin C. In a same way, skyrin act as a efficient radical scavenger as was detected by monitoring decreasing of the absorbance of galvanoxyl radical at their wavelength maxima vs time in the presence of this and also compared with other known anti-oxidants.

Singlet oxygen was quenching by addition of skyrin during the irradiation of rose Bengal as photosensitizer in an aqueous oxygenated media and detected by the histidine assay. The processes of singlet oxygen and radical hydroxyl scavenging can be related directly with the anti-oxidative processes of lipid peroxidation. Due to the protective effects on induced-lipid-peroxidation, the process of cell membranes peroxidation, it is possible that skyrin could play an important role in anti-oxidative skin toxicity. Skyrin may act as a protective substance in intrinsic aging or in induced extrinsic aging. Most of the naturally antioxidant compounds discussed in this work have not as yet been wholly screened for their effectiveness and safety for human use in clinical trials. It could be shown that skyrin is so effective that dipyridamole and vitamins C and E and that it is probably due to its radical scavenger properties. The reactive oxygen intermediates such as hydroxyl radical, hydrogen peroxide, superoxide anion and singlet oxygen are believed to play a major role in many pathological conditions including skin cancer. These intermediates are very short-lived species and can react with DNA protein and unsaturated fatty acids, thereby causing DNA strand breaks and oxidative damage as well as protein-protein DNA cross-links. It has been suggested that cancer-inducing damage to cells might be prevented by antioxidants through the scavenging of excess free radicals that are by-products of many normal metabolic functions, or by reducing the adverse effects of carcinogens or radiation. The now reported antioxidant activity and the investigated free radical scavenging property of skyrin may also play a role in its potential chemo-preventive property, but further investigations are needed to examine in more depth the mechanisms underlying its possible cancer preventive activity. Therefore, there is a need to continue the tests for these selected antioxidants and their combinations with these vitamins.

5. Conclusions

Skyrin is an efficient scavenger of free radical species ($^{\cdot}OH$, $R^{\cdot}$) and of singlet oxygen ($^1O_2$). The antioxidant activity of skyrin has been proved and compared with that of known antioxidants as emodin, dipyridamole, vitamins C and E in different chemiluminescence assays and in lipid peroxidation. The antioxidant behaviour of skyrin was very similar to that of emodin.

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