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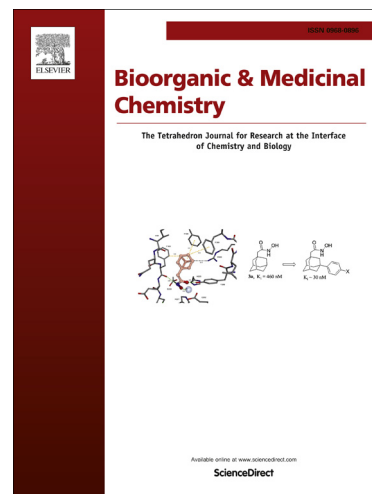
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Design, Synthesis and Biological Activity of a Novel Rutin Analogue with Improved Lipid Soluble Properties

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Abstract

Recent interest in flavonoids has increased greatly due to their biological and pharmacological activities. Flavonoids, consist of a large group of low molecular weight polyphenolic substances, naturally occurring in fruits, vegetables, tea, and wine, and are an integral part of the human diet. Rutin is a common dietary flavonoid that is widely consumed worldwide from plant-derived beverages and foods as traditional and folk medicine remedy as well. Rutin exhibit important pharmacological activities, including anti-oxidation, anti-inflammation, anti-diabetic, anti-adipogenic, neuroprotective and hormone therapy. Here, we present the synthesis, antimicrobial, antiproliferative and pro-apoptotic effect on human leukemic K562 cells of compound **R2**, a new semi-synthetic derivative of Rutin as compared to Rutin itself. The new derivative was also included in finished topical formulations to evaluate a potential application to the dermatology field in view of the antioxidant/antimicrobial/antinflammatory properties. Stability studies were performed by HPLC; PCL assay and ORAC tests were used to determine the antioxidant activity. **R2** presented an antioxidant activity very close to that of the parent Rutin while bearing much better lipophilic character. Regarding antiproliferative effects on the human K562 cell line, **R2** was found to be more effective than parent Rutin. Preliminary experiments demonstrated that **R2** inhibits NF-kB activity and promotes cellular apoptosis.

1. Introduction

In recent years, many herbs and natural compounds have increasingly been receiving public interest as complementary and alternative medicines [1]. World Health Organization (WHO) has urged the evaluation on the effectiveness of plant-based drugs due to the lack of scientific information [2]. The natural Rutin (3′,4′,5,7-tetrahydroxyflavone-3-rutinoside) is one of the most attractive phytochemicals because of its pharmacological activities. Therefore, it is considered as an important flavonoid in pharmaceutical industry [3].

Flavonoids are polyphenolic compounds that are found ubiquitously in plants. The flavonoids have aroused considerable interest because of their potential beneficial effects on human health; they have been reported to have antiviral, antiallergic, antiplatelet, anti-inflammatory, antitumor, prebiotic, antimicrobial and antioxidant activities [4-7]. The flavonoids consist of a large group of low molecular weight polyphenolic substances, naturally occurring in fruits, vegetables, tea, and wine, and are an integral part of the human diet.

Among them, Rutin (2-(3,4-dihydroxyphenyl)-4,5-dihydroxy-3-[3,4,5-trihydroxy-6-[(3,4,5-trihydroxy-6 methyl-oxan-2-yl)oxy-methyl]oxan-2-yl]oxy-chromen-7-one) also known as quercitin-3-rutinoside or sophorin, is a flavonol glycoside containing the flavonol quercitin and the disaccharide rutinose. It is consumed in fruits, vegetables and plant-derived beverages such as tea and wine. It is abundantly found and distributed in plants such as in buckwheat seed, fruits, and fruits rinds, especially citrus fruits (orange, grape-fruit, lemon). Rutin displays cardiac protective, cholesterol- and blood pressure-lowering effects [8], also helps inflammation recovery [9], arthritis [10], and cancers [11].

Natural products are frequently used for the development of novel actives to treat various bacterial infections. Different secondary metabolites such as flavonoids have shown significant antimicrobial properties [12]. Rutin has shown potent antimicrobial activity against a wide range of pathogens, i.e., bacteria, fungi, and viruses [13].

Rutin has significant range □scavenging properties on oxidizing species such as OH radical, superoxide radical, and peroxyl radical [14]. □ Rutin has been reported to have clinical relevant functions, including antihypertensive, antihemorrhagic activity, the strengthing of the capillaries of blood vessels, the regulation of the capillary permeability, and the stabilization of platelets [6,15]. The inflammatory response is also an important component in the pathogenesis of vascular injury and endothelial dysfunction is related especially to leukocyte recruitment during formation of the vascular inflammatory lesion [16,17]. Two important mediators are involved in vascular inflammatory processes: tumor necrosis factor-α (TNF-α) and NF-κB which are factors belonging to same strictly regulate pathways. For istance, NF-κB is a well known proinflammatory transcriptional factor that

is activated in response to proinflammatory cytokines such as TNF-α and interleukin 1β (IL-1β) [18,19]. There is considerable evidence suggesting that suppression of NF-κB signaling pathway confers significant vascular protective effects which delays or prevents vascular diseases in animal models of disease [19,20]. Therefore, preventing the production of TNF-α and/or inhibiting TNF-α mediated NF-κB activation in vascular endothelial cells are considered to be promising therapeutic targets for vascular inflammatory diseases. Taken together, these properties are potentially beneficial in preventing and reducing peripheral capillary problems, mainly involved in water retention and capillary fragility thus encouraging topical applications.

Beside these properties, the major disadvantage associated with this molecule is the limited bioavailability, connected with its low liposolubility, that limits its practical applications [21] in topical field.

Continuing our studies ☐on phytochemicals bearing phenylpropanoids structure [22,23], we modified Rutin in order to devise a semi-synthetic derivative liable to improve lipid solubility, while maintaining the characteristic polyphenol free hydroxyl moieties to express activity characteristic of the aglicone moiety. This properties are particularly interesting to the dermatology field, were biological properties of biophenols are often impaired by the poor lipophilicity, thus with difficulties in the distribution in topical formulation. For these reasons, Rutin and **R2** were investigated for their stability in finished topical formulations. The biological activity was evaluated by preliminary model assays suitable to give indications on interesting polyphenols activities, namely antioxidant, anti-inflammatory, anti-proliferation, apoptosis induction and ☐lastly antimicrobial infections that are ☐particularly sensitive to permeability of the cell ☐walls to antimicrobial agents [24,25].

2. Results and Discussion

2.1. Chemistry

Rutin was esterified at all hydroxyl moieties using propionyl chloride in presence of 4-dimethylaminopyridine (4-DMAP) and then selectively deacylated at the phenolic hydroxyl groups, using N,N,N-triethylamine (TEA) in methanol (Scheme 1). The selective removal of acyl groups from phenol moieties represented a difficult task because of the competition with the sugar one. After several trials satisfactory yields were obtained by the use of methanol in presence of

TEA at room temperature. The reaction conditions were also adapted to obtain consistent amount of **R2** necessary for the biology and formulation study.

Scheme 1 to be inserted here

2.1.2. Partition coefficient (LogP)

In order to evaluate changes in the lipophilic properties of the molecule induced by derivatization, the partition coefficient (LogP), calculated as the logarithm of the ratio of concentrations of the substance in octanol and water, was measured. This determination was conducted in a biphasic system of octanol (20 mL) and water (20 mL at pH 6) at 25 °C. After 30 min of stirring, the biphasic mixture was centrifuged for 10 min at 4,000 rpm to complete separation of phases and then 5 mL of each phase was used for spectrophotometric determination (at 360 nm) concentrations of Rutin (5 mg/20 mL) at the equilibrium. A similar analysis has been performed for **R2** (5 mg/20 mL) at 346 nm.

The data are described in Table 1 and show a significant difference in the distribution of **R2** and the parent rutine with "reversion" of the hydrophilic/lipophilic properties, for **R2** higher in octanol than in water, (1.09 LogP), whereas Rutin was much less concentrated in octanol than water with a LogP −0.64.

Table 1 to be inserted here

2.1.3. Antioxidant Activity

Rutin and **R2** were tested to determine their antioxidant capacity by PCL analysis and ORAC test. PCL data showed that Rutin and the semisynthetic derivative **R2** have a quite high antioxidant activity against superoxide anion (1883.08 and 1232.67 μmol Trolox/gr of product respectively) (Figure 1, Panel A). Indeed, lipidization consent to Rutin to maintain a very significant antioxidant activity that can be thus expressed also in lipids compartments.

Figure 1 to be inserted here

Moreover, ORAC test results show a high antioxidant activity with respect to the peroxyl radical, with better results seen for Rutin compared to **R2** (10708.06 and 728.61 μmol TE/gram product respectively). This large difference, is probably to ascribe to lipophilicity of **R2** because ORAC protocol is particularly suited for hydrophilic compounds and thus possible separation of the compound may occurs during the 24 h of analysis on the PBS acqueous medium.

2.2. Formulation Studies

We have shown recently, that the high potency of a molecule is useless if not expressed also when inserted within a formulation [26] thus, we have also investigated the antioxidant activity and the stability of the active components incorporated into finished dermo-cosmetic formulations at 0.3% (p/p) at starting and when subjected to accelerated aging at 40 °C.

Two different cosmetic vehicles were developed: Formulation **1** (using a cosmetic base, O/W, made with ingredients devoid of any antioxidant properties) and Formulation **2** (using a water free formula also devoid of antioxidant properties).

PCL analysis (at time zero) has confirmed the antioxidant activity of the formulation **1** containing b) Rutin and c) **R2** as compared to the c) cosmetic base (5.04, 3.81 and 0 nanomol Trolox/mg respectively) (Figure 1, Panel B).

To complete the study, formulations **1** and **2** containing the active ingredients, were submitted to accelerated aging in oven at 40 °C and monitored by HPLC to evaluate the stability of Rutin and **R2** in function of time. Quite unattended, Rutin resulted stable not only in the anhydrous formulation **2** but also in the O/W emulsion (formulation **1**) and even in both formulations over 150 days in the oven (data not shown). As concerns **R2**, we have observed, over 150 days at 40°C, that the derivatives is stable in both formulations (Figure 2). PCL analysis of the formulations **1** have shown maintenance of the anti-oxidant activity (Fig.1 Panel B).

Figure 2 to be inserted here

2.3. Antifungal Activity

Rutin and its derivative **R2** have been studied for their antifungal activities against nine fungal species [*Epidermophyton floccosum* (Hartz) Langeron e Milochevitch, *Trichophyton rubrum*

(Castellani) Saboraud, *Trichophyton tonsurans* Malmsten, *Trichophyton violaceum*, *Trichophyton mentagrophytes* (Robin) Blanchard, *Microsporum gypseum* (Bodin) Guiart e Grigorakis, *Nannizzia cajetani* Ajello, *Microsporum canis* Bodin, *Nannizzia gypsea* (Nann.) Stockd. (perfect state of *Microsporum gypseum)*] using the Sabouraud Dextrose Agar (SDA) diffusion method, in DMSO solvent (Table 2).

The antifungal activity of Rutin and its derivative **R2**, as shown in Table 2, did not give interesting outcomes: in fact, in all cases the percentages of inhibition are extremely low and the highest value was 15.8 % by **R2** on *Trichophyton violaceum* at the highest dose. In other cases the substance showed an hormone-like effect with a fungal growth higher than that of the controls (+).

Table 2 to be inserted here

2.4. Antiproliferative activity on human leukemic K562 cells

In order to assay the potential antiproliferative effects of Rutin and **R2** esapropionate derivative on human tumor cells, we have used the leukemic K562 cell line, which has been elsewhere published as a very useful system to screen for antitumor agents [27] . In order to analyze antiproliferative effects the cell number/ml was analyzed after three days of cell growth (when control cells are in the exponential phase of cell growth) in the presence of increasing concentration of Rutin and **R2** (from 0.5 μM to 1 mM). The results obtained indicate that the IC_{50} values for Rutin and **R2** are very different, showing that the **R2** derivative is more active than Rutin in inhibiting K562 cell proliferation (Table 3).

Table 3 to be inserted here

2.5. Inhibition of NF-kB/DNA Interactions

To verify a possible mechanism of action of **R2**, we analyzed by EMSA (Electrophoretic Mobility Shift Assay) the effects of both Rutin and **R2** on the in vitro biological activity of purified p50 subunit of NF-kB (Nuclear Factor-kappaB), since Rutin has been reported to have important effects on NF-kB regulated biological functions in several cellular systems. EMSA was performed allowing molecular interactions between the ^{32}P-labeled NF-kB oligonucleotide and the NF-kB p50

in the absence or in the presence of Rutin and **R2**. The obtained results, shown in Figure 3, demonstrate that Rutin and its derivative **R2** are both active in inhibiting the *in vitro* NF-kB/DNA interactions, with a similar efficiency. These data support the concept that **R2** can exert its effect on K562 cell proliferation through inhibition of the cell-cycle regulator NF-kB; in addition, the inhibition of NF-kB function might anticipate an effect of Rutin and **R2** on apoptosis, in consideration of the anti-apoptotic role of NF-kB in a variety of cellular model systems.

Figure 3 to be inserted here

2.6. Apoptosis on human leukemic K562 cells

In order to verify the effects of R2 on apoptosis, K562 cells were treated for 3 days with different concentrations (close to the IC_{50} values previously established and shown in Table 3) of Rutin and **R2**. Representative results are shown in Figure 4, and the full data obtained are summarized in Figure 5. Both derivatives produce pro-apoptotic effects on K562 cells when are used at concentrations approaching the IC_{50} values. However, R2 was found to be slightly more active than Rutin. The physiological level of apoptosis found in cultured untreated K562 cells was 11.37% (Figures 4-5). 500 and 1000 μM Rutin causes a slight pro-apoptotic effect (apoptotic cells: 13.20% and 14.75%, respectively); **R2**, instead, induces apoptosis more efficiently (apoptotic cells: 17.70% and 27.46%, at 5 and 10 μM respectively). No effects of Rutin were found at 5 and 10 μM (figure 4 and data not shown).

Figure 4 to be inserted here

Figure 5 to be inserted here

3. Conclusions.

Rutin and its derivative R2 were investigated to explore the effect on activity profile and possible topical applications of lipidization by a propionyl side chain. Very interestingly, R2 maintained similar antioxidant capacity to the parent Rutin in PCL analysis (lipophilic environment) and ORAC test (hydrophilic environment). This demonstrates that the structural modification does not decrease efficacy toward oxidative species. By the formulation study, we have demonstrated that the structural modification in fact consented to allow the incorporation of Rutin moiety into a lipophilic based topical formulation, but still keeping free the active phenol moieties. This consents to extend the activity of Rutin from a hydrophilic to a lipophilic compartment. In order to exploit functional effects, beside antioxidant ones, the antifungal activity of Rutin and its derivative R2 on dermatofites have been explored but they both show a very low inhibition. Because hyper-proliferation is involved in several cutaneous problems and diseases, preliminary antiproliferative effects on the human K562 cell line were investigated: R2 was found to be more effective than Rutin (Table 3); this is likely to be due to the increased solubility in lipid compartments. While final conclusion regarding the mechanism of action of R2 is far to be reached, preliminary experiments demonstrated that R2 inhibits NF-kB/DNA interactions (Figure 3) and promote cellular apoptosis (Figures 4 and 5). These observations are relevant, because, on one hand, NF-kB transcription factor is known to plays a crucial role in cell cycle and, on the other hand, induction of apoptosis is a well established anti-tumor strategy. However, the effects of R2 on apoptosis (which are significantly more pronounced than Rutin) cannot be explained simply by R2-mediated inhibitory effects on NF-kB/DNA interactions (which are very similar to those exhibited by Rutin). Accordingly, an extensive study should be undertaken to identify the molecular basis of the differences between Rutin and R2 in inducting pro-apoptotic effects. In this field of investigation, even focusing on Rutin-mediated effects, several different (but eventually synergistic) biological activities have been described. For instance, Rutin and its metabolites have demonstrated their capacity in reducing the activation of NF-κB signaling in several experimental systems, including lipopolysaccharide-activated macrophages inhibiting the I-κBα phosphorylation and degradation responsible of translocation of NF-kB from the cytoplasm to the nucleus [28,29]. In addition, Rutin was found to inhibit NF-kB gene expression, reducing the level of NF-kB protein [30]. Furthermore, Rutin might affect NF-kB also by alternative mechanism of action. For instance, Rutin was found to target EGFR kinase [31]. This is of interest, in consideration of the firmly demonstrated linkage between EGFR family receptors and NF-kB signaling [32,33]. In any case, whatever the mechanism of action will be found, R2 deserves further studies focusing on possible anti proliferative activity on other tumor cell lines.

Moreover, we like to underline that NF-kB is also responsible for inflammatory processes and alteration of its activity is associated with several human problems and pathologies, including osteoporosis, rheumatoid arthritis, and cancer. For istance, it is well known that NF-κB is a critical transcription factor responsible for inflammatory process in Cystic Fibrosis [34,35]. Therefore, targeting NF-κB appears to be a relevant therapeutic strategy, as previously described and demonstrated [36,37]. Our data strongly suggest that **R2** might be proposed as anti-inflammatory agent provide with better solubility in lipids and thus improved bioavalability in skin diseases and accelerated skin ageing such is photoageing [38,39].

4. Experimental

4.1. General

All reactives were from Sigma-Aldrch srl (Milan, Italy). Reaction course was routinely monitored by thin-layer chromatography on silica gel using precoated Macherey-Nagel Durasil-25 plates with detection under a 254-nm UV lamp and/or by spraying the plates with FeCl3 solution or potassium permanganate diluted solution. Column chromatography was performed with Macherey-Nagel 0.063–0.2 mm/70–230 mesh silica gel. The molecular weights of the compounds were determined by ESI (Micromass ZMD 2000), and the values are expressed as [MH]+. 1H-NMR spectroscopy was obtained using a Bruker AC-200, a Varian VXR-200 or a Mercury Plus400 spectrometer. HPLC analysis was performed using an Agilent 1100 Series HPLC System equipped with a G1315A DAD and with a Hydro RP18 Sinergi 80A column (4.6 × 150 mm, 4 μm) from Phenomenex. Phloridzin was purchased from Sigma Aldrich (Steinheim, Germany).

4.2. Synthesis of Rutin Decapropionate (R1)

To a pre-cooled (0 °C) solution of Rutin (400mg, 0.66 mmol) and DMAP (2.9 g, 23.74 mmol) in CH_2Cl_2 (50 mL), propionyl chloride (19.32 mmol) was slowly added. After stirring at room temperature for 14 h the reaction mixture was washed with H_2O, and with Brine. The organic phase was then dried (Na_2SO_4), filtered and the solvent evaporated under reduced pressure. Crystallization with Et_2O and petroleum ether, afforded **R1** (73% yield) as a white foam.

^{1}H-NMR (400 MHz, CDCl3): δ(ppm) 1.01-1.03 (m, 18H, 6xCH3); 1.08-1.11 (m, 6H, 2xCH3);1.12-1.18 (m, 9H, 3xCH3); 2.20-2.24 (q, 2H, CH2); 2.31-2.35 (m, 4H, 2xCH2); 2.36-2.45 (m, 6H, 3xCH2); 2.48-2.54 (m, 6H, 3xCH2); 2.64-2.68 (q, 2H, CH2); 2.95-3.26 (m, 2H, -OCH2-, sugar);

4.12-4.14 (m, 1H, sugar); 4.41-4.43 (m, 1H, sugar); 4.61-4.64 (t, 1H, sugar); 4.78-4.83 (m, 2H, sugar); 4.97-5.08 (m, 3H, sugar); 5.24 (s, 1H, sugar); 5.78 (s, 1H, sugar); 6.94-7 (m, 3H, aromatic); 7.17 (s, 1H, aromatic); 7.31-7.33 (d, 1H, aromatic). ^{13}C-NMR (400 MHz, CDCl3): δ 8.8, 8.47, 9.06, 9.14, 9.22, 9.34, 9.40, 9.45, 9.53, 9.65, 16.04, 27.42, 27.47, 27.52, 27.55, 27.57, 27.61, 27.63, 27.67, 27.69, 27.72, 67.81, 69.03, 69.21, 70.54, 70.62, 71.40, 71.61, 71.98, 72.43, 95.86, 99.10, 103.31, 106.6, 110.46, 116.50, 120.42, 124.26, 131.12, 135.35, 142.65, 144.8, 149.02, 151.53, 155.71, 156.68, 174.18, 174.24, 174.32, 174.39, 174.45, 174.53, 174.68, 174.72, 174.83, 174.96, 175.46; ESI MS: m/z 1172.3 Da [M+H]$^+$, $C_{57}H_{70}O_{26}$ Mol. Wt. 1171.15 calcd.

4.3. Synthesis of Rutin Hexapropionate (R2)

Et$_3$N (3.5 mL) was added to a solution of **R1** (500 mg, 0.43 mmol) in anhydrous MeOH (25 mL). The mixture was stirred at room temperature under argon atmosphere for 18 h and then neutralized by adding of formic acid (3.7 mL) at 0 °C. Next the mixture was evaporated under reduced pressure. The crude residue was dissolved in AcOEt and wash (water, Brine). The organic phase was than dried (Na$_2$SO$_4$), filtered and the solvent evaporated under reduced pressure. The residue obtained was purified by silica gel column chromatography (eluent: CH$_2$Cl$_2$/MeOH, 98/2, v/v), gave **R2** (41%) as yellow ocher foam.

^{1}H-NMR (400 MHz, DMSO-d6): δ(ppm) 0.8-1.2 (m, 21H, 7xCH$_3$); 2.08–2.16, (m, 4H, 2xCH$_2$); 2.20-2.38 (m, 4H, 2xCH$_2$); 2.60-2.62 (m, 2H, 1xCH$_2$); 3.58 -3.7 (m, 2H, -O-CH$_2$- sugar); 3.9-3.98 (m, 1H, sugar); 4.40-4.50 (m, 1H, sugar); 4.70-4.80 (m, 1H, sugar); 4.85-5.05 (m, 3H, sugar); 5.10-5.2 (m, 1H, sugar); 5.38-5.43 (m, 1H, sugar); 5.58-5.62 (m, 1H, sugar); 6.56-6.58 (d, 1H, sugar); 6.74-6.82 (m, 2H, aromatic); 7.4-7.42 (s, 1H, aromatic); 7.6-7.68 (m, 1H, aromatic); 9.42-9.5 (m, 2H, 1H aromatic, 1 OH); 10.95 (s, 1H, OH); 11.1 (s, 1H, OH); 12.48 (s, 1H, OH). ^{13}C-NMR (400 MHz, DMSO-d6): δ 8.55, 8.72, 8.86, 9.06, 16.82, 38.73, 39.87, 39.08, 39.3, 39.5, 39.71, 39.92, 40.12, 65.70, 66.52, 68.39, 68.5, 68.61, 69.75, 71.5, 71.74, 71.95, 97.33, 98.51, 98.66, 100.28, 108.51, 109.27, 115.19, 116.16, 120.55, 120.74, 134.48, 144.7, 148.32, 150.19, 154.67, 157.08, 162.06, 170.97, 172.15, 172.45, 172.59, 172.71, 172.84, 172.96; ESI MS: m/z 947.8 Da [M+H]$^+$, $C_{45}H_{54}O_{22}$ Mol. Wt. 946.9

4.4. Antioxidant Activity Assays

4.4.1. Photochemiluminescence (PCL) Method

PCL assay, based on the methodology of Popov and Lewin, [40] was used to measure the antioxidant activity of extracts with a Photochem® apparatus (Analytik Jena, Leipzig, Germany) against superoxide anion radicals generated from luminol, a photo-sensitizer, when exposed to UV light (Double Bore® phosphor lamp, output 351 nm, 3 mWatt/cm^2). The antioxidant activity was measured using both ACW (Antioxidant Capacity of Water soluble substance) and ACL (Antioxidant Capacity of Liposoluble substance) kits provided by the manufacturer designed to measure the antioxidant activity of hydrophilic and lipophilic compounds, respectively [41]. For ACW studies, the luminol reagent and Trolox work solution were freshly prepared according to the ACW protocol. The presence of Trolox (or any other antioxidants from the extracts) retarded luminescence for a period: hence, a lag time was noted before a signal was measured. The duration of the lag, which is calculated by the computer software from the first derivative of the detector signal at its turning point and intersection with the x-axis, was plotted against the concentration of Trolox added to the assay medium. The concentration of the added extract solution was such that the generated luminescence fell within the limits of the standard curve. Therefore, the lag time (seconds) for the ACW assay was used as the radical scavenging activity and the antioxidant capacity calculated by comparison with a Trolox standard curve and then expressed as micromoles of Trolox per gram of dry matter of red fibre. In ACL studies, the kinetic light emission curve, which exhibits no lag phase, was monitored for 180 s and expressed as micromoles of Trolox per gram of dry matter. The areas under the curves were calculated using the PCLsoft control and analysis software. As greater concentrations of Trolox working solutions were added to the assay medium, a marked reduction in the magnitude of the PCL signal and hence the area calculated from the integral was observed. This inhibition was used as a parameter for quantification and related to the decrease in the integral of PCL intensities caused by varying concentrations of Trolox. The observed inhibition of the signal was plotted against the concentration of Trolox added to the assay medium. The concentration of the added extract solution was such that the generated luminescence during the 180 s sampling interval fell within the limits of the standard curve. The extracts for ACW and ACL measurements were centrifuged (5 min at 16000 g) prior to analysis. The antioxidant assay was carried out in triplicate for each sample, and 20 μL of the diluted extract (1:40, v/v) in HPLC-grade water (ACW) or HPLC-grade methanol (ACL) was sufficient to correspond to the standard curve.

4.4.2.Oxygen Radical Absorbance Capacity (ORAC) Assay.

The ORAC assay was carried out on a Fluoroskan FL® ascent (Thermo Fisher Scientific, Inc. Waltham,MA) with fluorescent filters (excitation wavelength: 485 nm; emission filter: 538 nm). The procedure was based on that given by Hong, Guohua & Ronald (1996) as modified in our previous work [42]. Briefly, in the final assay mixture (0.2 mL total volume), fluorescein sodium salt (85 nM) was used as a target of free radical attack with 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) as a peroxyl radical generator. Trolox, a water-soluble analogue of vitamin E, was used as a standard control: a calibration curve was carried out with 10, 20, 30, 40, 50 μM solution. The tested compounds were dissolved in PBS and prepared immediately before the experiments. The fluorescence measurements, carried out at 37° C, were recorded at 5 min intervals up 30 min after the addition of AAPH. The ORAC values, calculated as difference of the areas under the quenching curves of fluoresceine between the blank and the sample, were expressed as Trolox equivalents (TE), pH = 7.4. All the experiments were performed with three replicates.

4.5. Stability Studies

4.5.1. Cosmetic Matrices

In this study, Rutin and **R2** in different formulations have been subjected to accelerated aging in an oven at 40 °C, and analyzed by HPLC with the aim to asses its content over time.

The study was carried out on two different formulations, containing 0.3% of the active ingredient.

The formulations tested are as follows:

1. INCI: Aqua, Glycerin, Glyceryl stearate, Ceteareth-20, Ceteareth-12, Cetyl palmitate, Cetearyl alcohol, Dimethicone, Caprylic/capric triglyceride, Dicapryl carbonate, 1,2-Hexanediol, 1,2-Octanediol, Tropolone.

From a technological point of view, it is an O/W formulation.

2. INCI: Triethylhexanoin, Methyl methacrylate crosspolymer, Glyceryl tribehenate/eicosadioate, Alcohol denat., di-C20-40 alkyl dimer dilinoleate. From a technological point of view it is an anhydrous formulation.

4.5.2. HPLC Methods

HPLC analysis was performed using an Agilent 1100 Series HPLC System equipped with a G1315A DAD and with an Hydro RP18 Sinergi 80A column (4.6 x 150 mm, 4 μm) from

Phenomenex. The mobile phase consisted of water (0.01 M H_3PO_4) (solvent A) and acetonitrile (0.01 M H_3PO_4) (solvent B).

1) Rutin: in this study, Rutin in formulations has been subjected to accelerated aging in an oven at 40 °C, and analyzed by HPLC with the aim to asses the content of this active ingredient over time. The determination was carried out under isocratic condition, A: 80 % / B: 20 %. Separation was monitored with absorbance detection at 360±8 nm. The flow rate was 1.2 ml/min, the injection volume was 5 μl.

2) **R2**: in this study, **R2** in formulations has been subjected to accelerated aging in an oven at 40 ° C, and analyzed by HPLC with the aim to asses the content of this active ingredient over time. The determination is carried out under isocratic condition, A: 37 % / B: 63 %. Separation was monitored with absorbance detection at 364±8 nm. The flow rate was 1.2 ml/min, the injection volume was 5 μl.

4.5.3. Statistical evaluations

Relative standard deviations and statistical significance (Student's t test; P≤0.05) were given where appropriate for all data collected. One-way ANOVA and LSD post hoc Tukey's honest significant difference test were used for comparing the bioactive effects of different samples. All computations were made using the statistical software STATISTICA 6.0 (StatSoft Italia srl).

4.6. Antifungal activity

4.6.1. Microorganisms.

Rutin and its derivative **R2** were tested on fungal species, pathogenic for animals and humans, such as some dermatophytes.

The dermatophytes used were *Nannizzia cajetani* Ajello, CBS 495.70 strain; *Epidermophyton floccosum* (Hartz) Langeron and Milochevitch, CBS 358.93 strain; *Trichophyton violaceum* Malmsten, CBS 459.61 strain; *Trichophyton tonsurans* Malmsten, CBS 483.76 strain, *Trichophyton mentagrophytes* (Robin) Blanchard, CBS 160.66 strain, *Microsporum canis* Bodin CBS 4727 strain, *Nannizzia gypsea* (Bodin) Guiart et Grigoraki CBS 286.63 purchased from Centraal Bureau voor Schimmelcultures (CBS), Baarn, The Netherlands; *Trichophyton rubrum* (Castellani) Sabouraud

IHME 4321 strain, *Microsporum gypseum* (Bodin) Guiart and Grigorakis IHME 3999 strain obtained from the Institute of Hygiene and Epidemiology-Mycology (IHME), Brussels, Belgium. The cultures were maintained in the laboratory as agar slants on a suitable culture medium, that is, on Sabouraud dextrose agar (SDA; Difco), for the dermatophytes.

4.6.2. Evaluation of Antifungal Activity

To evaluate antifungal activity, cultures of each fungus were obtained by transplanting mycelium disks, 10 mm in diameter, from a single culture in stationary phase. These were incubated at 26 ± 1 °C on the medium suitable for each organism (SDA), on thin sterile sheets of cellophane, until the logarithmic phase of growth was reached. Then the fungi were transferred to Petri dishes containing the medium supplemented with the compound to be tested. Each compound was dissolved into dimethyl sulfoxide (DMSO), and a proper dilution was aseptically added to the medium at 45 °C to obtain a final concentration of 20, or 100 μg mL-1. The DMSO concentration in the final solution was adjusted to 0.1%. Controls were set up with equivalent quantities (0.1%) of DMSO. The growth rate was determined by measuring daily colony diameter for 7 days after the transport of the fungus onto dishes containing the substance to be tested. At this time the percentage growth inhibition in comparison with the control was evaluated for each fungus. Three replicates were used for each concentration. The percentage of growth inhibition was expressed as the mean of values obtained in three independent experiments.

The relative inhibition rate of the circle mycelium compared to blank assay was calculated via the following equation:

$$\text{Relative inhibition rate (\%)} = [(\text{dex} - \text{dex'})/\text{dex}] \times 100\%$$

where dex is the extended diameter of the circle mycelium during the blank assay; and dex' is the extended diameter of the circle mycelium during testing.

4.7 Proliferation assay

In standardized conditions, the antiproliferative effects of Rutin and **R2** were determined in human leukemic K562 cells, derived from a patient with chronic myeloid leukemia, were cultured in a humidified atmosphere of 5% CO_2/air in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Analitical de Mori, Milan, Italy), 50 units/mL penicillin, and 50 mg/mL streptomycin. Cell growth was studied by determining the cell number per

milliliter with a ZF Coulter Counter (Coulter Electronics, Hialeah, FL). Treatment with Rutin and **R2** was carried out by adding the appropriate drug concentrations at the beginning of the experiments (cells were usually seeded at 20000 cells/mL) [43]. The medium was not changed during the treatment time. The cell number/mL was determined as IC_{50} after 3 days of culture when untreated cells are in log phase of cell growth.

4.8 Apoptosis assay

K562 cells were treated with Rutin or **R2** at different time scales and concentrations. Cell viability and pro-apoptotic effects were measured by Annexin V and Dead cell assay with "Muse" method (Millipore Corporation, Billerica, MA, USA), according to the instructions supplied by the manufacturer. This procedure utilizes Annexin V to detect PS (Phosphatidyl Serine) on the external membrane of apoptotic cells. A dead cell marker was also used as an indicator of cell membrane structural integrity. It is excluded from live, healthy cells, as well as early apoptotic cells. Four populations of cells can be distinguished in this assay. Cells were diluted (1:2) with the one step addition of the "Muse" Annexin V and Dead Cell reagent [35]. After incubation of 20 min at room temperature in the dark, samples were analyzed. Data were acquired and recorded utilizing the Annexin V and Dead Cell Software Module (Millipore).

4.9 Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assays were performed by using double-stranded ^{32}P-labeled oligonucleotides as target DNA. Binding reactions were set up as described elsewhere [35,44] in binding buffer (10% glycerol, 0.05% NP-40, 10mM Tris-HCl pH 7.5, 50mM NaCl, 0.5mM DTT, and 10mM $MgCl_2$) and 0.25 ng of labeled oligonucleotide, in the presence of Rutin and R2 at different concentrations. After 20 min binding at room temperature, 0.1 μL/20μL of NF-kappaB-p50 (50 gsu) (PROMEGA, Madison, WI, USA) was added, in a total final volume of 20 μL. After the second binding reaction (for additional 20 min, at r.t.), samples were electrophoresed at constant voltage (200V for 30 min) through low ionic strength (0.25Å~TBE buffer) (1Å~ TBE/40.089M Tris-borate, 0.002M EDTA) on 6% polyacrylamide gels until tracking dye (bromophenol blue) reached the end of a 16 cm slab. Gels were dried and exposed for autoradiography with intensifying screens at -80°C. In these experiments, DNA/protein complexes migrate through the gel with slower efficiency. In studies on the inhibitors of protein/DNA interactions, addition of the reagents

was as follows: (i) labeled oligonucleotides mimicking the binding sites for TF to be modulated; (ii) active principles; (iii) binding buffer; and (iv) nuclear factors. The nucleotide sequences of double-stranded target DNA utilized in these experiments were 5'-CGC TGG GGA CTT TCC ACG G-3' (sense strand, NF-kappaB). The synthetic oligonucleotides utilized in this study were purchased from Sigma-Genosys (Sigma-Genosys, Cambs, UK).

Acknowledgements

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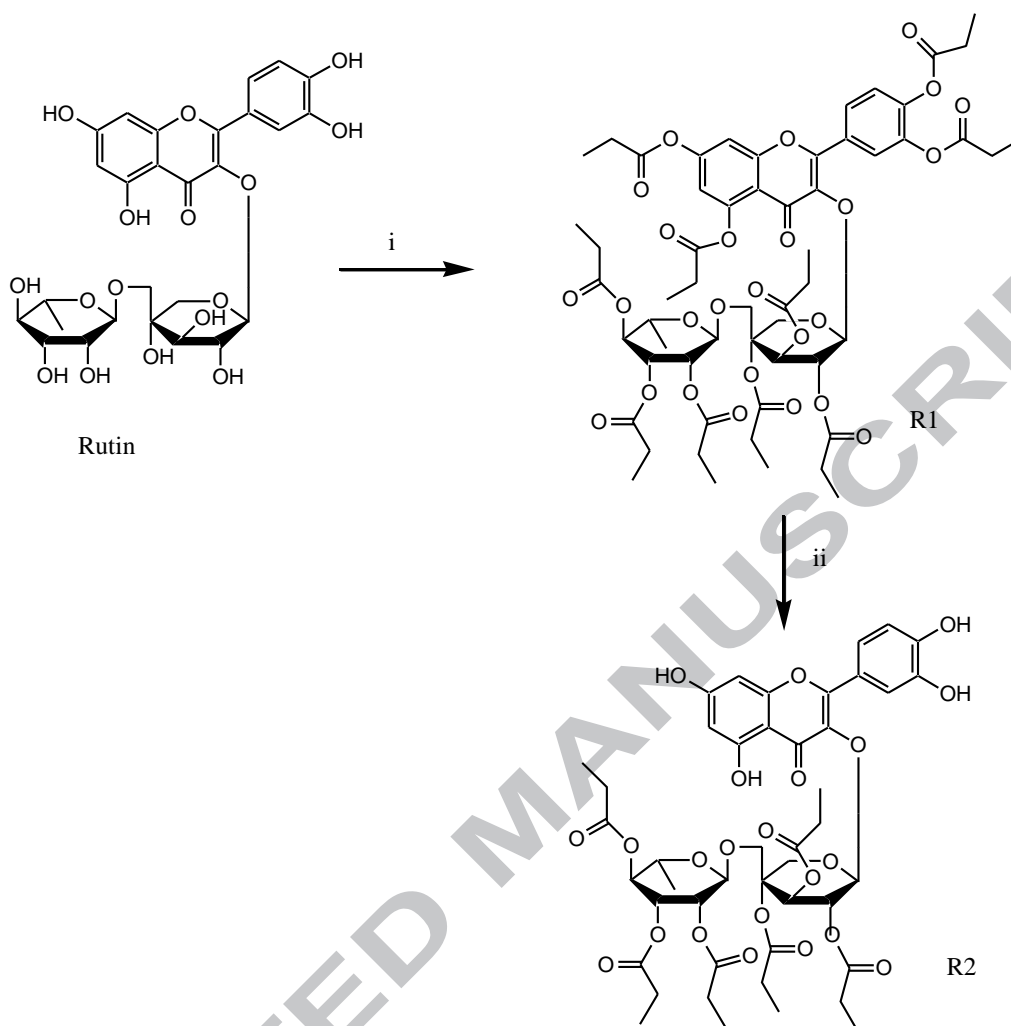
Scheme 1. Synthesis of the hexa-substituted derivative of Rutin **R2**. Reagents: i: DMAP, Propionyl Chloride, CH₂Cl₂, ii: TEA, MeOH.

Table 1. Partition coefficients of Rutin and **R2** in a biphasic mixture of octanol and water.

	Octanol (mg/20ml)	H_2O (mg/20 mL)	LogP *
Rutin	0.91	4.0	-0.64
R2	4.63	0.37	1.09

* LogP = log10 [mg/20 mL]octanol/[mg/20 mL]H_2O

Table 2. Growth inhibition following treatment with the two pure standards Rutin hydrate and its derivative **R2**.

Fungus	% Growth inhibition on the 7th days by Rutin conc. (μg/mL) of		% Growth inhibition on the 7th days by R2 conc. (μg/mL) of	
	20	100	20	100
Epidermophyton floccosum	1.9 ± 0.1	2.2 ± 0.3	+	+
Trichophyton rubrum	6.1 ± 0.4	9.9 ± 0.8	3.5 ± 0.9	4.3 ± 0.3
Trichophyton tonsurans	0	2.8 ± 0.5	5.4 ± 1.1	17.8 ± 2.2
Trichophyton violaceum	2.1 ± 0.2	4.2 ± 0.4	10.7 ± 0.6	15.8 ± 1.8
Trichophyton mentagrophytes	0	3.3 ± 0.4	0	5.7 ± 0.7
Microsporum gypseum	+	4.1 ± 0.2	5.4 ± 0.3	9.9 ± 0.9
Nannizzia cajetani	3.3 ± 0.1	12.3 ± 1.1	0.6 ± 0.2	3.5 ± 1
Microsporum canis	+	+	+	0
Nannizzia gypsea	+	+	1.9 ± 0.4	7.9 ± 0.7

Table 3. Effects of Rutin hydrate and **R2** on cell proliferation of K562 cells. The data represent the average ± SD of three independent experiments.

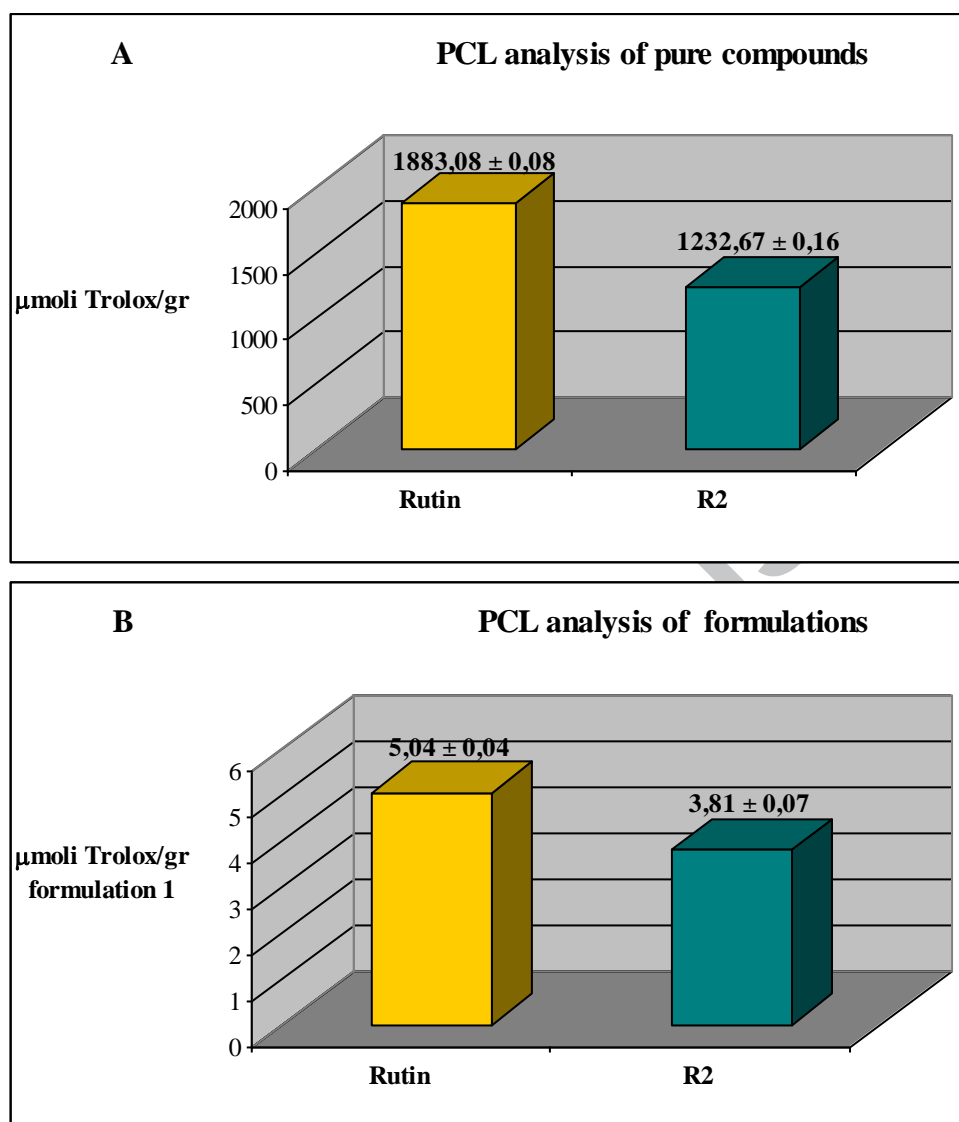| Cell line	IC_{50} (μM) of	
	Rutin	**R2**
K562	897 ± 43	7.17 ± 0.6

Figure 1. Panel **A**: PCL analysis of Rutin and R2. Panel **B**: PCL analysis of Rutin and R2 formulations. Each value was obtained from three experiments (Mean ± SE).
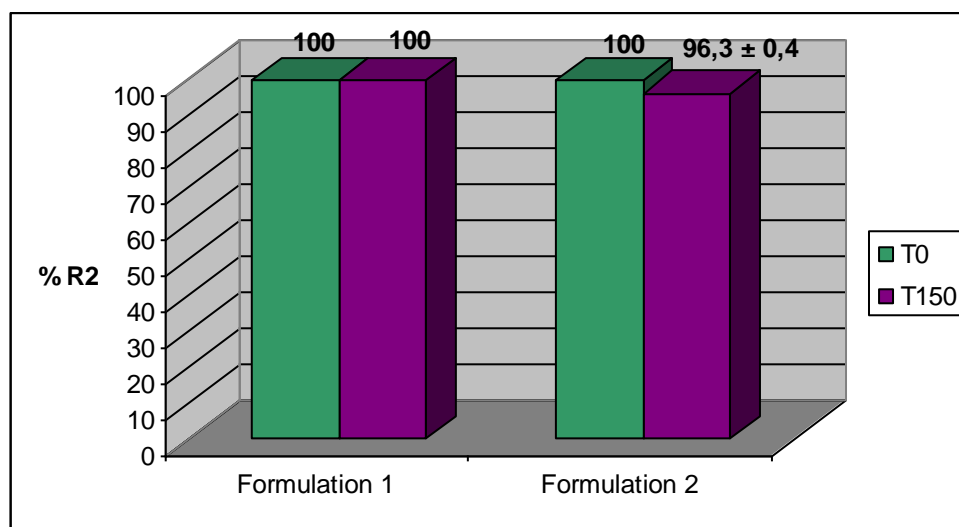
Figure 2. R2 recovery rate in the different formulations stored at 40 °C. Each value was obtained from three experiments (mean ± SE).
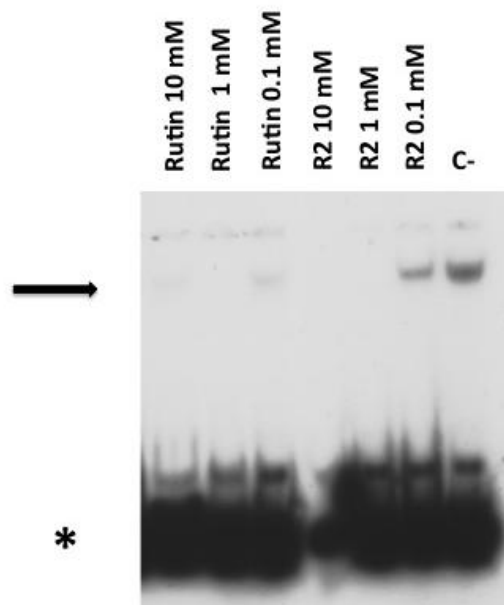
Figure 3. Effects of Rutin and **R2** on the molecular interactions betweeb NF-kB p50 and ^{32}P-labeled target NF-kB double-stranded oligonucleotide. Compounds were first incubated with NF-kB, and then the ^{32}P-labeled target NF-kB oligonucleotide was added. NF-kN/DNA complexes were analyzed by polyacrylamide gel electrophoresis. Arrow indicates NF-kB/DNA complexes; asterisk indicates the free ^{32}P-labeled target NF-kB probe.
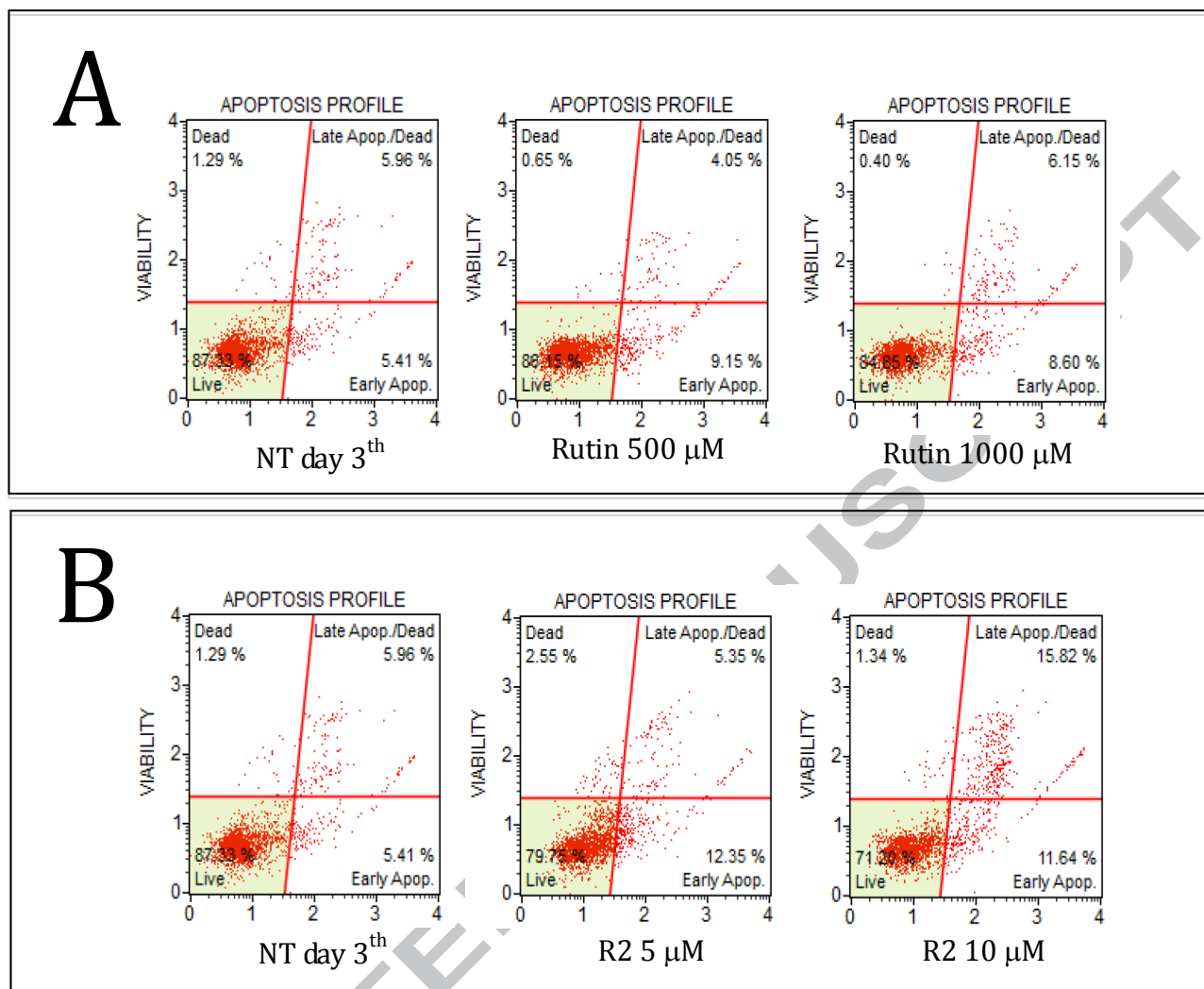
Figure 4. Apoptosis profile of K562 cells treated for 3 days with Rutin (**Panel A)** and **R2** derivative **(Panel B)** at the indicated concentrations. NT: untreated K562 cells.
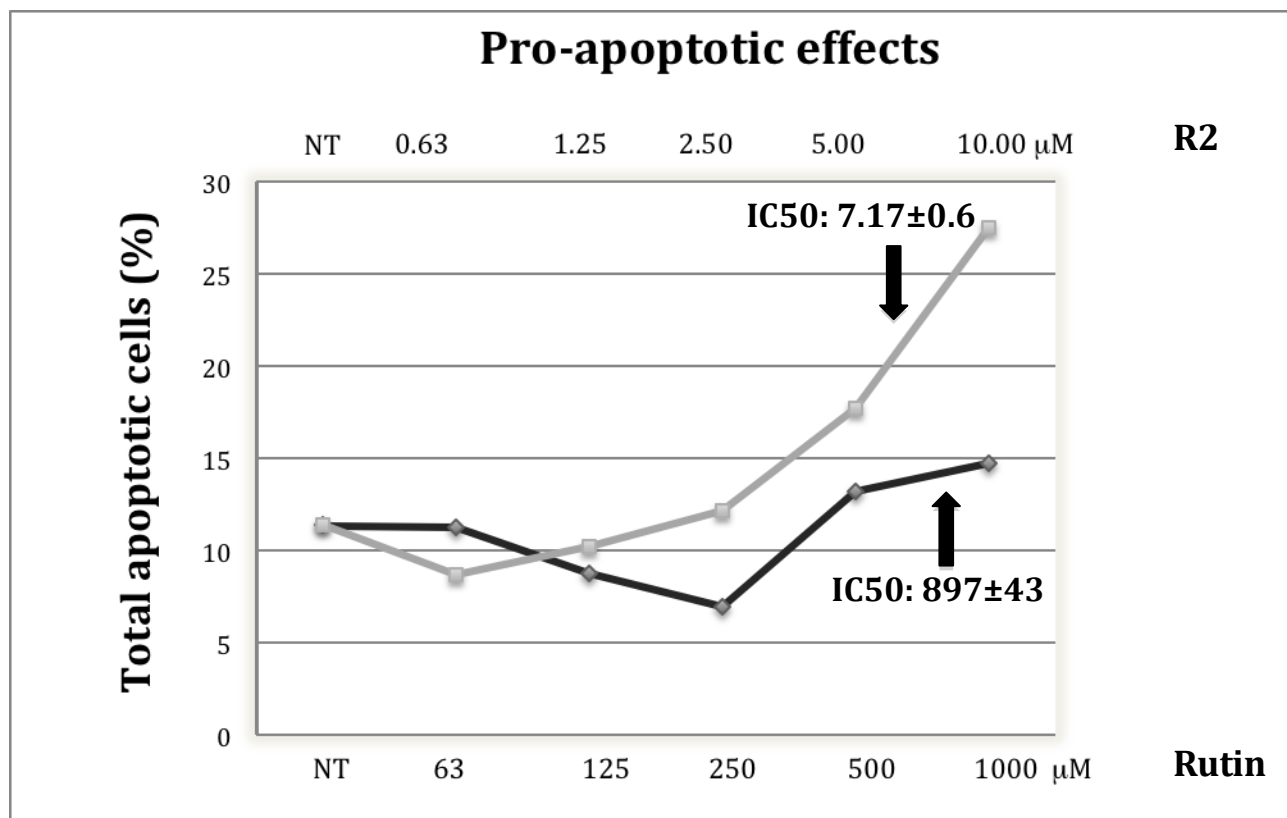
Figure 5. Induction of apoptosis following treatment for 3 days with different concentrations of K562 cells with Rutin (black line) and R2 (grey line). NT: untreated control K562 cells. The arrows indicate the IC_{50} concentrations for Rutin and **R2**.

Graphical Abstract

i: DMAP, Propionyl Chloride, CH$_2$Cl$_2$, ii: TEA, MeOH.