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Investigation of the Antioxidant Activity of African Potato (*Hypoxis hemerocallidea*)

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African potato (AP) is widely used as an immune booster for the treatment of various ailments. The norlignan glycoside hypoxoside, a major phytoconstituent of AP, its aglycon rooperol, and an aqueous preparation of lyophilized AP corms were screened for in vitro antioxidant activity using the DPPH (1,1-diphenyl-2-picryl hydrazine) and FRAP (ferric reducing ability of plasma) tests. Inhibition of quinolinic acid (QA) induced lipid peroxidation in rat liver tissue was studied in vitro using the thiobarbituric assay (TBA). Superoxide free radical scavenging activity was determined by the nitroblue tetrazolium assay. An isocratic HPLC method was developed to quantitatively determine both hypoxoside and rooperol concurrently. While rooperol and AP extracts reduced QA-induced lipid peroxidation in rat liver homogenates and significantly scavenged the superoxide anion at pharmacological doses, in comparison, hypoxoside was virtually devoid of activity. Since hypoxoside is converted to rooperol in vivo following administration of AP, the results indicate that the hypoxoside component in AP could have value as an antioxidant prodrug.

KEYWORDS: African potato; HPLC; prodrug; hypoxoside; rooperol; DPPH assay; FRAP assay; lipid peroxidation

INTRODUCTION

Hypoxis hemerocallidea [African potato (AP)], also known as *Hypoxis rooperi* (*Hypoxidaceae*), has a long history of traditional use for a diversity of ailments (*1*) and more recently has been the subject of several scientific studies (*2*). In many parts of Africa the corms of this attractive yellow flowered herb have been used in the treatment of urinary diseases, prostate hypertrophy, and internal cancer (*3*).

The characteristic secondary metabolite of the plant is hypoxoside (**Figure 1**), whose common name is (*E*)-1,5-bis (4′-*β*-D-glucopyranosyloxy-3′-hydroxyphenyl)pent-4-en-1-yne (CAS Reg No: 83643-94-1), a norlignan diglucoside present in the corms of this plant (*3*). This compound has an uncommon aglycon structure consisting of a C6 (aromatic)−C3−C2−C6 (aromatic) skeleton. The glycoside has low toxicity and the corm containing it is also used as food (*4*). As shown in **Figure 1**, following the administration of AP, the hypoxoside contained therein is converted in the human body by colonic bacterial *β*-glycosidase to rooperol (*3*, *5*). Pharmacokinetic studies have indicated that rooperol can be found in feces, and metabolites are found in the serum and urine as its glycosides, sulfates, mixed glucuronides, and sulfuronides (*6*). These metabolites, when deconjugated back to rooperol, were found to be cytotoxic

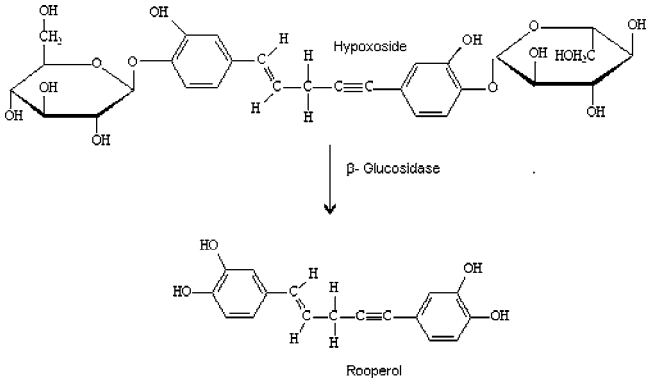

Figure 1. The structures of hypoxoside and rooperol.

to cancerous cells (*7*). Research conducted over many years has shown that rooperol has potent pharmacological activity, whereas hypoxoside acts as a nontoxic, multifunctional prodrug (*8*). Furthermore, rooperol has a structural resemblance to nordihydroguairetic acid (NDGA), a known strong antioxidant, and gave comparable results in the inhibition of leukotriene synthesis in the polymorphonuclear leukocyte and prostaglandin synthesis in platelet microsomes (*5*, *9*). Like NDGA, rooperol was found to interact with the oxidative process in human blood. Rooperol and NDGA differ from each other only in the C-bridge connecting the two catechol molecules. Rooperol has a pent-4-en-1-yne-diyl bridge, whereas NDGA, a potent antioxidant,

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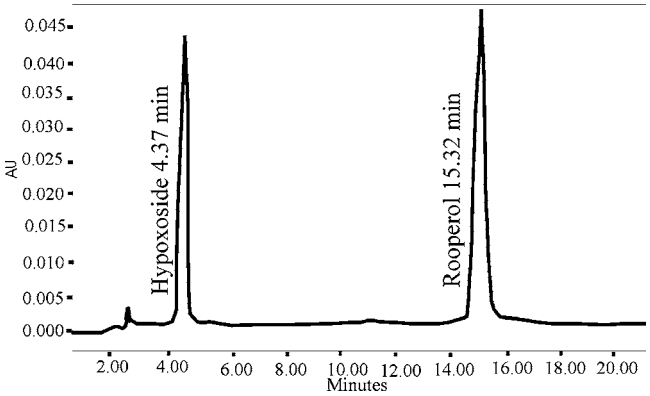

Figure 2. HPLC chromatogram of hypoxoside and rooperol.

has a 2,3-dimethyl-1,4-butanediyl bridge. It has been shown that there is a significant difference in the resonance structure of the biologically active semi-quinone radicals of rooperol and the known antioxidant NDGA, which makes rooperol and its glycoside, hypoxoside, potential candidates for antioxidant studies (*5*, *9*). Steenkamp et al. (*10*) demonstrated significant hydroxyl ion scavenging ability with aqueous and ethanolic extracts of AP using a Fenton-type reaction that was measured by the spin trapping method (*11*).

Recently, there has been an increased interest in antioxidants that have the ability to scavenge free radicals, such as superoxide radicals, hydroxyl radicals, and others that have been implicated in a number of degenerative diseases, including cancer (*12*), cardiovascular disease (*13*), cataracts (*14*), macular degeneration (*15*), impaired wound healing (*16*), gastrointestinal inflammatory diseases (*17*), and other inflammatory processes. In view of the possibility that rooperol should possess antioxidant activity, its glycoside, hypoxoside, is also likely to have some antioxidant activity.

In the present study, we investigated the potential antioxidant activity of an aqueous preparation of lyophilized AP and independently assessed its major phytochemical constituent, hypoxoside, as well as the latter's aglycon, rooperol. Since no HPLC analytical method has previously been published for the simultaneous qualitative analysis of hypoxoside and rooperol in a plant extract using an isocratic mobile phase, an additional objective of this study was to develop a simple and reliable HPLC method to detect these compounds in the lyophilized corms of AP in order to confirm their presence in the samples tested.

MATERIALS AND METHODS

Reagents. DPPH (1,1-diphenyl-2-picryl hydrazine), TPTZ (2,4,6-tripyridyl-s-triazine), QA (quinolinic acid), BHT (butylated hydroxytoluene), TBA (2-thiobarbituric acid), NBD (nitroblue diformazan), NBT (nitroblue tetrazolium), 1,1,3,3-tetramethoxypropane (99%), quercetin, and ascorbic acid were obtained from Sigma Chemical Co (St. Louis, MO). Glacial acetic acid, sodium acetate, and ferric chloride were of analytical grade (BDH chemicals, Poole, UK), and HPLC grade solvents were purchased from Romil LTD (The Source, Water Beach, Cambridge, UK). TCA (trichloroacetic acid), KCN (potassium cyanide), ethanol, and butanol were purchased from Saarchem, Johannesburg, South Africa. Water was purified in a Milli-Q system (Millipore, Bedford, MA), and all samples were filtered using Durapore (PVDF) 0.45 μm membranes purchased from the same source.

Plant Material and Phytochemicals. The corms of AP were collected on Settler's Hill adjoining the botanical garden of Rhodes University, Grahamstown, South Africa. A voucher specimen (AP 08/2003) was submitted to the herbarium, Faculty of Pharmacy, Rhodes University. Hypoxoside was isolated by a procedure developed in our laboratory and was characterized before usage, and its purity was confirmed chromatographically and by NMR spectroscopy (*18*). A sample of crystalline rooperol (99.0%), characterized by chromatography and mass spectrometry, was gratefully received from Dr. Carl Albrecht, CANSA, South Africa.

Table 1. Comparison of Antioxidant Activities Using Quercetin as Control (DPPH Assay)

sample	concn (μg/mL)	% inhibn of DPPH at 20 min mean ± SD
aqueous AP preparations	100	12.75 ± 0.40
	500	37.87 ± 3.31
	1000	66.43 ± 1.09
hypoxoside	32	–
rooperol	8	29.22 ± 5.21
	16	49.97 ± 2.17
	32	80.32 ± 0.22
quercetin control solutions	8	25.73 ± 5.21
	16	49.73 ± 2.17
	32	87.62 ± 0.78

Table 2. Comparison of Antioxidant Activities Using Ascorbic Acid as Control (FRAP Assay)

sample	concn (μg/mL)	FRAP μmol/L at 20 min mean ± SD
aqueous AP preparations	100	89.81 ± 2.57
	500	429.71 ± 9.24
	1000	803.81 ± 11.21
hypoxoside	32	–
rooperol	8	259.16 ± 7.21
	16	490.66 ± 14.69
	32	860.82 ± 7.14
ascorbic acid control solutions	8	173.56 ± 2.42
	16	288.56 ± 4.85
	32	471.01 ± 7.26

Sample Extraction. Freshly collected corms of AP were thoroughly washed under running water. They were then sliced into uniform cubes of approximately 0.5 cm^3 and flash-frozen with liquid nitrogen before being lyophilized for 12 h in vacuo. The lyophilized material (freeze-dried African potato, FDAP) was milled and sieved (40 mesh size) and aqueous preparations were made by weighing out ~1 g of the powdered material and extracting with hot water. The mixture was vortexed for 1 min and then sonicated for a predetermined optimized time of 20 min and subsequently centrifuged (1000*g*) for 2 min. The supernatant was filtered through Durapore (PVDF) filters and lyophylized prior to use in preparing desired concentrations for the investigations.

Preparation of Hypoxoside and Rooperol Solutions. A stock solution of 5 mg/mL of hypoxoside was prepared with Milli-Q water at room temperature, whereas the stock solution of rooperol (2.5 mg/mL) was made at 60 °C to facilitate its solublization. Both the solutions were sonicated for 2 min before being serially diluted to the desired concentrations.

Chromatographic Analysis. The analysis was carried out on an Alliance 2690 HPLC system (Waters Corp., Milford, MA) equipped with a 2996 photodiode array (PDA) detector, degasser, column heater and autosampler. A Synergi Hydro RP column (4 μm, 4.6 × 50 mm i.d.) (Phenomenex, Torrance, CA) was used at 23 ± 2 °C. Separation was achieved using a mobile phase consisting of methanol:water (48:52) at a flow rate of 1 mL/min in isocratic mode. The temperature was maintained at 23 ± 2 °C, and 10-μL samples of each reference compound, hypoxoside and rooperol, were injected. The eluate was monitored by UV detection at a wavelength of 260 nm, and hypoxoside and rooperol peaks were identified by spectral analysis using a photodiode array (PDA) detector.

Free Radical Scavenging Ability. The DPPH radical scavenging activity of the samples was determined using the method proposed by

McCune and Timothy (*19*). The extinction of the purple color of the DPPH due to the presence of an odd electron gives a strong absorption band at 517 nm. When this free electron gets paired off in the presence of a free radical scavenger, the absorption decreases and the resulting decolorization process is stoichiometric.

To 1.5 mL of DPPH (0.0394 mg/mL in methanol) was added 0.25 mL of test solution. The decrease in absorbance was measured after incubation for 20 min. The aqueous AP preparation (0.1, 0.5, 1.0 mg/mL), hypoxoside (32 μg/mL), and rooperol (4, 8, 16, 32 μg/mL) were compared to quercetin solutions (4, 8, 16, 32 μg/mL), a well-known antioxidant (*19*). Experiments were conducted in quadruplicate and are represented as the percentage (%) inhibition of the DPPH radical.

Determination of Ferric Reducing Activity of Plasma (FRAP). FRAP was used to determine the total antioxidant potential of the samples (*20*). In this assay the electron-donating capacity of the antioxidant was measured by the change in absorbance at 593 nm when a blue-colored Fe^{2+}-tripyridyltriazine (Fe^{2+}TPTZ) compound is formed from a colorless oxidized Fe^{3+} form. Calibration curves were prepared from aqueous solutions of $FeSO_4$ at different concentrations ranging from 100 to 1000 μM/L. Working reagent (0.9 mL) (25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution) was mixed with 30 μL of each of the aqueous AP preparations (0.1, 0.5, 1 mg/mL) and 90 μL of distilled water, and the change in absorbance was used to calculate the antioxidant efficiency of the test samples.

The results from the aqueous AP preparations of hypoxoside (32 μg/mL) and rooperol (4, 8, 16, 32 μg/mL) were compared to the antioxidant activity of ascorbic acid (8, 16, 32 μg/mL) at 20 min. Care was taken that all the solutions were diluted to fit within the linearity range and were used on the same day of preparation. These experiments were also conducted in quadruplicate.

Animal Care and Homogenate Preparation. Adult male Wistar rats, weighing between 250 and 300 g, were purchased from the South African Institute for Medical Research (Johannesburg, South Africa). The animals were housed in a controlled environment with a 12-h light−dark cycle and were given access to food and water ad libitum. The Rhodes University animal ethics committee approved protocols for the experiments. For the purpose of lipid peroxidation and superoxide anion assays, rats were sacrificed by cervical dislocation and the livers were removed and perfused with saline. Livers were homogenized (10%, w/v) in 0.1 M phosphate-buffered saline (PBS), at pH = 7.4 and used immediately for the assay.

Lipid Peroxidation Assay. The elevated amount of known end products of lipid peroxidation in animal material is probably the evidence most frequently quoted in support of free radical induced tissue damage (*21*). The thiobarbituric acid test is the most widely used assay for measuring lipid peroxidation (*21*) and involves the reaction between malondialdehyde (MDA), an end product of lipid peroxidation, and thiobarbituric acid to yield a pink chromogen, which is measured colorimetrically at 532 nm using a spectrophotometer (*21*).

Lipid peroxidation was determined according to a modified method of Placer et al. (*22*). Homogenate (1 mL) containing 1 mM QA alone or in combination with either hypoxoside (12.5, 25, 50 μg/mL), rooperol (7.5, 15, 30 μg/mL), or aqueous AP preparations (1.25, 2.5, 5.0 mg/mL) was incubated at 37 °C in a shaking water bath for 1 h. At the end of the incubation period, 0.5 mL of BHT (0.5 mg/mL in ethanol) and 1 mL of 25% TCA were added. To avoid adsorption of MDA to insoluble proteins, the tubes were sealed and heated for 10 min in a boiling water bath to release protein bound MDA. The samples were cooled to 4 °C and centrifuged at 2000*g* for 20 min, and thereafter, 2 mL of the protein-free supernatant was removed from each tube, and 0.5-mL aliquots of 0.33% TBA were added to this fraction. All tubes were heated for 1 h at 95 °C in a water bath. After cooling, the TBA−MDA complexes were extracted with 2 mL of butanol. The absorbance was read at 532 nm and MDA results were determined from a standard curve generated from 1,1,3,1-tetramethoxypropane. Final results were presented as nmol of MDA/mg of tissue.

Superoxide Anion Assay (Nitroblue Tetrazolium Assay). A modification of the assay procedure of Das et al., which involves the reduction of the NBT dye by the superoxide anion to the insoluble NBD, was employed (*23*). NBD was extracted with glacial acetic acid and measured colorimetrically at 560 nm (*23*−*25*). It is known that cyanide is an inhibitor of complex IV of the mitochondrial electron transport chain, and distal inhibition of the electron transport chain by cyanide augments reactive oxygen species production (*26*). The primary mechanism of action of cyanide involves the inhibition of cytochrome oxidase a_1a_3, the terminal oxidative enzyme of the electron transport chain (*27*, *28*). These electrons have the capacity to leak out of the mitochondria and come in close proximity to O_2, resulting in the generation of the superoxide anion.

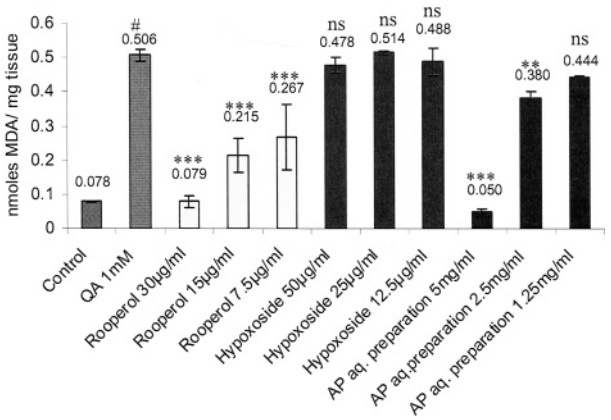

Figure 3. Effect of rooperol, hypoxoside, and AP on quinolinic acid (QA) induced lipid peroxidation in rat liver homogenates. Each bar represents the mean ± SD (n = 4); #p < 0.001 compared to control. ns = not significant compared to 1 mM QA induced lipid peroxidation, whereas ** and *** indicate significant at p < 0.01 and p < 0.001, respectively.

Homogenate (1 mL) containing KCN (1 mM) alone or in combination with either hypoxoside solutions (12.5, 25, 50 μg/mL), rooperol solutions (7.5, 15, 30 μg/ mL), or aqueous AP preparations (1.25, 2.5, 5.0 mg/mL) were incubated with 0.4 mL of 0.1% NBT in a shaking water bath for 1 h at 37 °C. Termination of the assay and extraction of NBD was carried out by centrifuging the samples for 10 min at 2000*g* followed by resuspension of the pellets with 2 mL of glacial acetic acid. The absorbance was measured at 560 nm and converted to micromoles of diformazan using a standard curve generated from NBD. Final results are expressed as μmol of diformazan/mg of tissue.

Statistical Analysis. All experimental data were analyzed using the one-way analysis of variance (ANOVA), and significant differences among the means from quadruplicate analysis at (p < 0.05) were determined by the Student−Newman−Keuls multiple range test.

RESULTS AND DISCUSSION

The 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay was used to examine free radical scavenging activity. This method is useful to determine the hydrogen-donating capacity of a molecule (*29*−*32*), but not any possible reactions with free radical intermediates and production of oxidative chain reactions. The ferric-reducing ability of plasma (FRAP) assay was utilized to examine nonenzymatic antioxidant capacity using a redox-linked colorimetric method (*20*). The TBA test was used to assess the extent of lipid peroxidation, which leads to cell death due to damage caused to membranes by reactive oxygen species (ROS) (*21*, *33*). Since superoxide ($O_2^{\bullet -}$), which can be generated through multiple enzymatic and nonenzymatic pathways, is often the start of the oxidative stress cascade (*34*−*36*), the NBT assay was used to investigate superoxide anion free radical scavenging activity (*23*−*25*, *35*, *36*). Each of these above tests provides a different insight on potential antioxidant activity and used in tandem are extremely valuable to comprehensively assess such activity.

Qualitative Analysis of Phytochemicals by an Isocratic HPLC Method. The method gave well-resolved peaks of both hypoxoside and rooperol in a single run. Retention times were

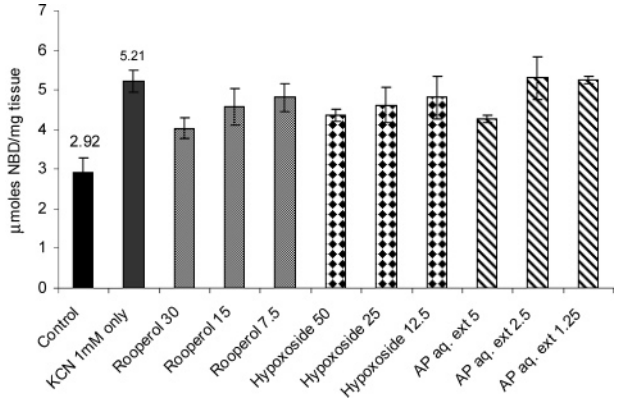

Figure 4. Effect of rooperol, hypoxoside, and aqueous extract AP on 1 mM KCN-induced super oxide radical in rat liver homogenate. Each bar represents the mean ± SD ($n = 4$); #$p < 0.001$ compared to control. ns = not significant compared to 1 mM KCN induced group, whereas ** and *** indicate significant at $p < 0.01$ and $p < 0.001$, respectively.

4.34 and 15.32 min, respectively (**Figure 2**). The limits of detection (LOD) and limits of quantification (LOQ) of both hypoxoside and rooperol were 1 and 4 μg/mL, respectively. The FDAP was extracted separately with methanol as well as with warm (60 °C) water and was subjected to analysis using the developed HPLC method. Both extracts of FDAP, at 500 μg/mL concentration, did not show the presence of rooperol. This was expected, since rooperol does not exist in free form unless the hypoxoside undergoes enzymatic hydrolysis in the presence of β-glycosidases in the gastrointestinal tract (*3*, *5*). The amount of hypoxoside in FDAP was determined as 10.2% (dry weight, w/w).

Free Radical Scavenging. In the present study the free radical scavenging effects of the aqueous AP preparation, hypoxoside, and the aglycon of hypoxoside, rooperol, were compared with that of quercetin. (**Table 1**). The free radical scavenging activity of the aqueous AP preparation using the DPPH assay showed high levels of activity that increased with increasing concentration. Rooperol showed free radical scavenging activity comparable to the same quercetin concentrations, suggesting that it is a relatively potent free radical scavenger at higher concentrations. Hypoxoside, even at a high concentration of 32 μg/mL, showed no significant free radical scavenging activity.

Determination of Ferric Reducing Activity of Plasma (FRAP). Ferric reducing activity plots were highly linear ($r^2 > 0.999$). As shown in **Table 2**, the Fe^{2+} formed by the aglycon rooperol at equivalent ascorbic acid concentrations was significantly ($p < 0.001$) higher than that produced by the standard. At 20 min, the aqueous AP preparation at 1 mg/mL gave a FRAP value of approximately 804 μmol/L compared to 471 μmol/L, which resulted at the highest ascorbic acid concentration of 32 μg/mL. Since hypoxoside, considered to be the main component in AP, did not show any significant antioxidant activity in this assay, the relatively low antioxidant activity observed at the highest concentration of aqueous AP preparation could be due to some other unidentified chemical components present in the AP preparation, such as, for example, ubiquitous plant phenolic components.

Effect of AP Extract on Lipid Peroxidation. Quinolinic acid chelates ferrous ions and it is the QA−iron complex that generates free radicals and acts as a pro-oxidant by initiating lipid peroxidation (*37*). The present study demonstrated that the aqueous AP preparations at concentrations of 2.5 and 5 mg/mL and also rooperol at 7.5, 15, and 30 μg/mL significantly ($p < 0.001$) reduced QA-induced lipid peroxidation in liver homogenates, with rooperol being the more potent antioxidant. It was noted that at a concentration of 30 μg/mL, rooperol shows no significant difference from the control, which means it completely abolished the QA-induced lipid peroxidation (**Figure 3**). This could be through scavenging the free radicals that are formed or by interacting with the ferrous ions, thereby preventing the formation of the QA-ferrous ion complex (*38*). However, hypoxoside, even at a higher concentration (50 μg/mL), did not reduce the QA (1 mM) induced lipid peroxidation.

Effect of AP Preparations on Superoxide Anion Generation. The results show that only the higher concentrations of the AP preparation ($p < 0.01$), hypoxoside ($p < 0.01$), and rooperol ($p < 0.001$) significantly scavenge the superoxide anions, thereby curtailing the reduction of NBT to NBD (**Figure 4**).

In conclusion, the objective of these studies was to assess the antioxidant activity of the main phytochemical constituent of AP, hypoxoside, as well as its aglycon, rooperol. Hypoxoside, did not show any significant antioxidant whereas, rooperol, which is not present in AP per se, was shown to have significant antioxidant activity. The latter is probably due to the dicatechol-like structure similar to the known antioxidant compound, nordihydroguairetic acid. Interestingly, the aqueous AP preparations showed increased free radical scavenging activity with higher concentrations, whereas the hypoxoside constituent present in the AP preparations, when tested alone, did not show antioxidant activity. This could be due to various other phytochemicals present in AP. Hence, aqueous preparations of AP have good potential for increased antioxidant activity in vivo since, in addition to AP's confirmed in vitro antioxidant activity, the rich inactive content of hypoxoside (10.16%) is readily converted to rooperol in the gastrointestinal tract of humans. These features indicate that AP could have value as an antioxidant prodrug.

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