In vitro anticancer and antioxidant activity studies on Drosera peltata J.E.Sm.

Drosera peltata J.E.Sm.’in in vitro antikanser ve antioksidan aktivitesi

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ABSTRACT

Aim: The present study aimed to assess the in vitro antioxidant and anticancer properties of Drosera peltata J.E.Sm.

Methods: Antioxidant capacity of the extracts were tested on hydroxyl, DPPH, super oxide, nitric oxide and ABTS radicals; of ferrous ion chelating ability and by reducing power assay. Ascorbic acid was used as the standard antioxidant for the above all assay methods. Dalton’s Ascitic Lymphoma (DAL) and Ehrlich Ascitic Carcinoma (EAC) were used as cell lines to assess its in vitro anticancer effect. Trypan blue dye and LDH leakage assays were carried out to know its anticancer potential. Moreover, total flavonoid content was estimated to find out the exact reason for its antioxidant potential.

Results: The aqueous extract (AEDP) in comparison with the ethanol extract (EEDP), showed considerable antioxidant and anticancer activities in all the models tested. The minimum IC$_{50}$ values of 48.87 ± 0.53 μg/ml was shown by EEDP in metal chelating assay and 28.46 ± 0.76 μg/ml by AEDP in hydroxyl radical scavenging assay. Higher doses of both extracts (250μg/ml) showed significant cytotoxic effects on DAL and EAC of LDH leakage assay model. Total flavonoid contents of both extracts were estimated and expressed as mg/g quercetin equivalent (QE), where ethanol extract was rich in flavonoid content than aqueous extract.

Conclusion: These study results were concluded that D. peltata exhibited excellent antioxidant activity against freeradicals which were generated from different models and showed very good anticancer activity against the two cell lines, this results might be due to the phytoconstituent such as flavonoid present in the plant extracts.

Keywords: Drosera peltata; in vitro; Antioxidant; Anticancer; Total flavonoid content; Dalton’s Ascitic Lymphoma (DAL); Ehrlich Ascitic Carcinoma (EAC).

ÖZET

Amaç: Bu çalışmanın amacı, Drosera peltata J.E.Sm.’nin in vitro koşullarda antioksidan ve antikanser etkinliğini değerlendirilmektir.


Bulgular:Etanol ekstraktıyla (EEDP) kıyaslandığında su lake ekstrakt (AEDP) daha fazla antioksidan ve antikanser etkinlik göstermektede idi. EEDP ile minimum IC$_{50}$ değeri 48.87 ± 0.53 μg/ml ile metal yakalama testinde elde edildi. AEDP ile minimum IC$_{50}$ değeri ise 28.46 ± 0.76 μg/ml ile hidroksil yakalama testi ile elde edildi. Her iki ekstraktta yüksek dozları (250 μg/ml) LDH sızıntı testi ile gösterilen sitotoksit testinde DAL ve EAC hücreleri üzerine sitotoksik etki gösterdi. Her iki ekstraktta toplam flavanoid miktarı mg/g quercetin ekivalan (QE) olarak tafe edildi ve etanol ekstraktının flavanoid miktarı su lake ekstrakt daha fazla idi.


Anahat kelimeler:Drosera peltata; in vitro; Antioksidan; Antikanser; Total flavanoid miktarı; Dalton’s Ascitic Lymphoma (DAL); Ehrlich Ascitic Carcinoma (EAC).

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INTRODUCTION

Plants with phenolic and flavonoid compounds like secondary metabolites have been reported to be excellent antioxidant and anticancer drugs [1]. The various plants under the genus *Drosera* (sundew plant) belong to Droseraceae family. The plants belonging to the *Drosera* genus are insectivorous plants consisting of approximately 170 species [2]. In India, *Drosera peltata* J.E.Sm., *Drosera indica* L. and *Drosera burmannii* Vahl have been reported. These species are used as important constituents in the Ayurveda preparation such as *Swarnabhasma* (Golden ash) [3]. Different species of *Drosera* are used in the treatment of various ailments in Homeopathic system of medicine [4]. Chloroform extracts of *D. peltata* showed bactericidal effect on *Streptococcus, Prevotella*. This is attributed to plumbagin which is the main active compound of the extract [5]. A pigment which is yellowish brown crystalline from *D. peltata* is used for dyeing silk [6].

The aim of present study is to evaluate the anticancer and antioxidant potential of the ethanol and aqueous extracts of *D. peltata* using various antioxidant in vitro assays.

MATERIALS AND METHODS

**Plant material**

The whole plant of *Drosera peltata* J.E.Sm. was collected from Munnar hills, Kerala, India during July 2011. It was identified and authenticated by Prof. Madhava Chetty, K, Taxonomist, S.V. University, Tirupati, Andhra Pradesh, India. The whole plants become washed; shade dried, powdered and stored in a container for experiments.

**Preparation of the extracts**

**Alcohol extract**

A weighed amount of the air-dried powdered drug changed into extracted with ethanol (90 %v/v) in a Soxhlet equipment. The extract became concentrated by using a rotary flask evaporator where the temperature no longer exceeding 50°C. The ethanol extract (EEDP) was suspended in distilled water for experimental use.

**Aqueous extract**

The marc from the ethanol extract was macerated with chloroform- water for 24 h to obtain AEDP (aqueous extract of *Drosera peltata*). AEDP was concentrated under vacuum and dissolved in distilled water for experimental studies. Both the extracts were stored in air tight containers.

**In vitro antioxidant activity**

All the assays methods under this study were done in triplicate [3].

**DPPH (1, 1 diphenyl 2, picryl hydrazyl) radical scavenging**

To 1mM of methanol solution of DPPH, add equal volume of the extracts dissolved in alcohol was added at different concentrations like 5, 10, 20, 40, 80, 160, 320 and 640 mcg/ml and made up to a final volume of 1.0 ml. Instead of extract, an equal amount of alcohol was taken as control. After 20 min incubation, absorbances were read at 517 nm.

Inhibition (%) = \( \frac{(A_0 - A_1)}{A_0} \times 100 \)

Where \( A_0 \) is absorbance of control and \( A_1 \) is absorbance of test.

**Hydroxyl radical scavenging activity**

This technique involves generation of hydroxyl radicals the usage of Fe³⁺/ ascorbate/ EDTA/ H₂O₂ gadget by way of Fenton reaction. Formaldehyde was formed upon the reaction between hydroxyl radicals with DMSO (dimethyl sulphoxide). The formaldehyde turned into combined with Nash reagent (2M ammonium acetate with 0.05M acetic acid and 0.02M acetyl acetone in distilled water). The concentration of the yellow color developed was measured at 412 nm the usage of a spectrophotometer, towards reagent blank.

HRSA (%) = 1 - (Differences in absorbance of sample/Difference in absorbance of blank) \times 100

**Nitric oxide radical scavenging**

Sodium Nitroprusside 5mm became prepared in phosphate buffer pH 7. 4. To 1 ml of various concentrations of extract, Sodium Nitroprusside 0.3 ml was added and tubes were incubated at 25°C for five h after, in which Griess reagent (0.5 ml) was added. The absorbance became read at 546 nm.

Inhibition (%) = \( \frac{(A_0 - A_1)}{A_0} \times 100 \)

Where \( A_0 \) is absorbance of control, \( A_1 \) is test absorbance.

**Metal chelating activity**

EEDP and AEDP of concentrations of 5, 10, 20, 40, 80, 160, 320 and 640 mcg/ml were added to 1 ml of 2 mM FeCl₂. By adding 1 ml of Ferrozine (5 Mm), the reaction was initiated and absorbance was read at 562 nm after 10 min.

Inhibition (%) = \( \frac{(A_0 - A_1)}{A_0} \times 100 \)

Where \( A_0 \) is control absorbance, \( A_1 \) is test absorbance.
Reducing power Assay

1 ml of extracts were mixed with 2.5 ml of 200 mM phosphate buffer (pH 6.6) and then added 30 mM of potassium ferric cyanide (2.5 ml). These solution mixtures were incubated at 50°C for 20 min. Thereafter, 600 mM trichloroacetic acid (2.5 ml) was added and centrifuged for 10 min at 3000 rpm. The top layer of solution became mixed with equal volume (2.5 ml) of distilled water and FeCl3 (6 mM, 0.5 ml) were added and absorbance becomes measured at 700 nm.

Inhibition (%) = \( \frac{A_0 - A_1}{A_0} \times 100 \)

Where \( A_0 \) is control absorbance, \( A_1 \) is test absorbance.

Superoxide scavenging

The superoxide anion radicals had been generated in a aggregate containing of NBT (0.3 mM, 0.5 ml), 0.5 ml of Tris-HCl buffer (16 mM, pH 8, 0.5 ml), 1.0 ml extract and 1 ml Tris- HCl buffer (16 mM, pH 8, 0.5 ml). The reaction turned into began via the addition of PMS (Phenazine methosulphate 0.5 ml) to the mixture, which become then incubated at 25°C for 5 min and the absorbance become measured at 560 nm in opposition to a blank pattern.

Inhibition (%) = \( \frac{A_0 - A_1}{A_0} \times 100 \)

Where \( A_0 \) is control absorbance, \( A_1 \) is test absorbance.

ABTS radical scavenging assay

To the response mixture containing ABTS radical 0.3 ml, phosphate buffer (1.7 ml) and extracts (0.5 ml) have been added at different concentrations ranging from 5 to 640 μg/ml. Absorbance have been recorded at 734 nm, against reagent blank.

Inhibition (%) = \( \frac{A_0 - A_1}{A_0} \times 100 \)

Where \( A_0 \) is control absorbance, \( A_1 \) is test absorbance.

In vitro anticancer activity

Cytotoxicity effect of both ethanol and aqueous extracts were assessed by Trypan blue exclusion assay [7] and Lactate dehydrogenase (LDH) leakage assay [8].

Trypan blue exclusion assay

Different concentrations (5, 10, 50, 100, 150 and 250 mcg/ml) of EEDP and AEDP were prepared. In a test tube, 100 μl of extracts were mixed with 100 μl of 0.4% w/v trypan blue solution, diluted with 800 μl of sterile phosphate buffer saline. The mixture thus obtained was mixed with 100 μl cell suspensions (1×10⁶ in 1ml) of Dalton’s Ascitic Lymphoma (DAL) and Ehrlich Ascitic Carcinoma (EAC) and immediately loaded into the Neubauer Chamber. The stained and unstained cells were counted in all 8 fields each consisting of 16 squares under a microscope. Viability of cells were calculated as follows:

% Viability = \( \frac{\text{Number of stained cell}}{\text{Total number of cell}} \times 100 \)

Lactate dehydrogenase (LDH) leakage assay

As per the LDH cytotoxicity detection kit manual, from Sigma Aldrich Inc., USA, LDH leakage assay was conducted. In order to determine IC₅₀, 100 μl of DAL and EAC cell line at the concentration of 1×10⁶ cell/ml in a one of a 96 well plates with various concentration of extracts. This kit was incubated at 37°C for four h in 5% CO₂ atmosphere. This test was conducted in a triplicates manner and the cytotoxicity was calculated as follows,

% Cytotoxicity = \( \frac{\text{At}-\text{Ac}}{\text{Ac}} \times 100 \)

Where, At- Test sample Absorbance Ac- Absorbance of Control

Total flavonoid content

Total flavonoid content present in both extracts has been predicted by means of the usage of AlCl₃ [9]. The total flavonoid found in each extract was expressed as mg/g (QE). Quercetin is used as standard; various concentrations of 2, 5, 10, 15 and 20 μg/mL were used to get a standard graph. To 0.5 mL of these diluted quercetin solution, 1.5 mL of methanol, 0.1 mL of CH₃COOK 1M, 2.8 mL of H₂O & 0.1 mL of AlCl₃ (10% in distilled water) was added. The final reaction mixtures were thoroughly mixed and kept for 30 min at 32°C. After incubation the absorbances were read at 415 nm by using a UV spectrophotometer. In test solution 0.5 mL extracts were added instead of standard similarly, for blank, 0.1 mL of distilled water was added instead AlCl₃. Standard calibration curve for Quercetin was graphed by various concentrations at X axis and absorbance at Y axis.

Statistical Analysis

All experiments in in vitro antioxidant and anticancer were performed in triplicate manner and the results were presented as mean± standard deviation (SD).

RESULTS

The free radicals were scavenged by the extracts in a concentration based way in all in vitro models. Concentrations of 5, 10, 20, 40, 80, 160, 320 and 640 mcg/ml of the ethanol and aqueous extracts of D. peltata were tested for their antioxidant potency in different in vitro models and IC₅₀ values were presented in a Table 1.
**Table 1. In vitro antioxidant effect of EEDP and AEDP on various assay methods**

<table>
<thead>
<tr>
<th>Test extract/drug</th>
<th>DPPH Assay</th>
<th>ABTS Assay</th>
<th>Super oxide Assay</th>
<th>Nitric oxide Assay</th>
<th>Reducing power Assay</th>
<th>Hydroxyl radical Scavenging Assay</th>
<th>Fe2+ Chelating Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEDP</td>
<td>188.3±</td>
<td>144.6±</td>
<td>89.6±</td>
<td>65.4±</td>
<td>98.64±</td>
<td>55.2±</td>
<td>48.87±</td>
</tr>
<tr>
<td></td>
<td>0.13</td>
<td>0.17</td>
<td>0.85</td>
<td>0.16</td>
<td>0.58</td>
<td>0.42</td>
<td>0.53</td>
</tr>
<tr>
<td>AEDP</td>
<td>197.3±</td>
<td>291.73±</td>
<td>69.48±</td>
<td>73.18±</td>
<td>156.85±</td>
<td>28.46±</td>
<td>110.5±</td>
</tr>
<tr>
<td></td>
<td>1.84</td>
<td>0.83</td>
<td>0.8</td>
<td>1.5</td>
<td>0.12</td>
<td>0.7</td>
<td>0.87</td>
</tr>
<tr>
<td>Ascorbic acid (AA)</td>
<td>170±</td>
<td>110.12±</td>
<td>160.78±</td>
<td>85.71±</td>
<td>95.56±</td>
<td>76.8±</td>
<td>110.03±</td>
</tr>
<tr>
<td></td>
<td>1.42</td>
<td>0.18</td>
<td>0.26</td>
<td>0.82</td>
<td>0.78</td>
<td>0.35</td>
<td>1.02</td>
</tr>
</tbody>
</table>

The scavenging effect on DPPH radical increased with the raise in concentration of EEDP and AEDP. The percentage inhibition of EEDP was varying from 8% with 5 mcg/ml of the extract to 94% with 640 mcg/ml of extract. Similarly the percentage inhibition of AEDP was varying from 2% with 5 mcg/ml of the extract to 83% with 640 mcg/ml of extract. The IC$_{50}$ value of the EEDP and AEDP was calculated to be 188.3 ± 0.13 and 197.3 ± 1.84 mcg/ml while the ascorbic acid was 170 ± 1.42 mcg/ml.

The hydroxyl radical scavenging activity also increased with respect to increase in concentrations of the extracts. The IC$_{50}$ values of EEDP and AEDP were found to be 55.2 ± 0.42 and 28.46 ± 0.7 mcg/ml respectively, while that for ascorbic acid was 76.8 ± 0.35 mcg/ml. Both the extracts consumed lesser concentration than ascorbic acid in scavenging hydroxyl radical.

Similarly in NO scavenging assay, both the extracts consumed lesser concentration than the standard antioxidant ascorbic acid. The percentage of inhibition in NO scavenging assay was maximum (98%) at 640 mcg/ml of EEDP and 71% at AEDP. The IC$_{50}$ value of the EEDP and AEDP was calculated to be 65.4 ± 0.16 and 73.18 ± 1.5 mcg/ml while that for standard ascorbic acid was 85.71 ± 0.82 mcg/ml.

In reducing power assay, in presence of EEDP and AEDP, Fe$^{3+}$/ ferricyanide complex is reduced to the ferrous form. The IC$_{50}$ values of EEDP (98.64 ± 0.58) was greater than that of AA (95.56 ± 0.78) but lesser than that of AEDP (156.85 ± 0.12), indicating that EEDP has more reducing power than AEDP.

The absorbance of ferrozine-Fe2+ complex reduced with respect to concentration of extracts. Upon the reduction of the redox potential, in such a way that chelating effect with metal ion which are effective like secondary antioxidant and thereby stabilize the oxidized form of the metal ion. In the present work, lesser concentration of EEDP was consumed (48.87 ± 0.53) than AA (110.03 ± 1.02), whereas AEDP (110.5 ± 0.87) showed similar inhibition as that of standard AA.

Super oxide free radicals were scavenged with the increase in the concentration of EEDP and AEDP and moreover lower concentration of both extracts were devoted for this assay. The IC$_{50}$ were found to be 89.6 ± 0.85 mcg/ml for EEDP and 69.48 ± 0.8 mcg/ml for AEDP, whereas for standard AA it was 160.78 ± 0.26 mcg/ml. In super oxide free radical scavenging assay, AEDP showed lower IC$_{50}$ than EEDP but was more potent than standard AA.

In ABTS assay, presence of extracts showed significant inhibitory concentration with EEDP (144.6 ± 0.17) comparable with that of AA (110.12 ± 0.18) whereas AEDP showed an inhibitory concentration of 291.73 ± 0.83.

**In vitro anticancer activity of EEDP and AEDP against the test cell lines DAL and EAC by trypan blue dye exclusion and LDH leakage assay methods** are shown in Figures 1 and 2 respectively.

![Trypan blue assay](image)

**Figure 1.** Effect of EEDP and AEDP against cell line in trypan blue exclusion assay.
DAL and EAC. The inhibition concentration was compared with that of control. A dose dependent increase in the % of LDH leakage was observed. A maximum leakage (98% & 94%) of LDH was observed at a concentration of 250 mcg/ml. The % of LDH release was increased with increasing concentration of EEDP which is in direct proportion to the cell death (Figure 2).

**Figure 2.** Effect of EEDP and AEDP against cell line in LDH leakage assay methods

Total flavonoid content in EEDP and AEDP were calculated and presented as quercetin equivalent (QE) in Table 2. Similarly data were analyzed by calibration curve method, where, \( y = 0.0261x + 0.03404 \), \( r^2 = 0.9989 \), \( n = 6 \) was the calibration equation for quercetin. Based on linear regression analysis, the absorbance related concentration ranges (10, 7.5, 5.0, 2.5 and 1 μg/mL) also linear. The quantification range was from 2.2031 ± 0.021 to 10.5004 ± 0.02 μg/mL for EEDP and 0.6296 ± 0.022 to 5.9334 ± 0.01 for AEDP (absorbance from 0.0978 to 0.3140 for EEDP and 0.0568 to 0.1950 for AEDP) were calculated from the calibration curve. The straight line graph was shown in Figure 3.

**Figure 3.** Calibration curve of Quercetin

<table>
<thead>
<tr>
<th>Concentration of extracts μg/mL</th>
<th>Total flavonoid content in mg/g (QE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEDP</td>
<td>AEDP</td>
</tr>
<tr>
<td>100</td>
<td>0.242±</td>
</tr>
<tr>
<td>250</td>
<td>0.465±</td>
</tr>
<tr>
<td>500</td>
<td>0.598±</td>
</tr>
<tr>
<td>750</td>
<td>0.896±</td>
</tr>
<tr>
<td>1000</td>
<td>1.051±</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Antioxidants are also widely used as dietary supplements within the wish of keeping fitness and preventing sicknesses which includes most cancers and coronary cardiac diseases. Generated or formed free radicals causes oxidation of other micro molecules which can be controlled or avoided with the aid of number antioxidants substances which includes flavonoids, butylated hydroxyl anisole and many others [10].

The free radicals which are having unpaired electrons and are unstable, becomes stable after extracting electrons from other molecules of biological system. In continuation of the earlier reports, that is anticancer and antioxidant potential of other Indian Drosera species, an attempt was carried out on the third Drosera species in India such as *D.peltata* [3]. The anticancer and antioxidant activities could probably be due to the presence phytochemicals like flavonoids, phenolics etc in these species.

DPPH (1 - 1 diphenyl - 2 - picryl hydrazine), a stable free radical, get reduced by the addition of antioxidants via accepting either an electron or hydrogen radical. The amount of discoloration, that's from red to yellow, suggests the scavenging activity of the drug [11]. In a concentration related scavenging effects had been seen with 5 – 640 mcg /ml of extracts which was measured as change in intensity of yellow color in each test sample.

Griess reagent is used to estimate NO radicals, is unstable and reacts with oxygen molecule to give stable nitrate and nitrite. Suppression of released NO endorsed to direct NO scavenging, which was
increased with respect to raise in extract concentration [12]. In the present study, both the extracts inhibited the release of nitric oxide by their antioxidant potential which also a concentration dependent where EEDP showed better effect than the AEDP.

Reducing power of EEDP and AEDP was compared with ascorbic acid where the reducing capacity was assessed as the power to convert ferric to ferrous (reducing activity) which was indicated by the change in intensity of blue color [13]. In the present study, the extracts showed significant reducing power which was increasing with the increase in concentration of the sample from 5–640 mcg/ml.

Super oxide anion is a radical that could generate numerous reactive species which include hydrogen peroxide, hydroxyl radical and singlet oxygen, able to causes oxidative damage, in DNA,proteins, and in lipids [14]. Consequently, studying the scavenging effect of plant extracts on superoxide radical will clarify the mechanism of antioxidant effect. This study results revealed that, both the extracts were potent antioxidants than ascorbic acid.

The 2,2'-azinobis (3-ethyl benzthiazoline-6-sulfonic acid) (ABT+) cation radical can react towards most antioxidants including phenolics, thiols and Vitamin C, ABTS+, is also stable and the antioxidant potential of test samples inversely proportional to the absorbance of the radical cation [15].Scavenging activity of ABTS+ radical by EEDP and AEDP was found to be appreciable; this implies that the plant extract may be useful for treating radical-related pathological damage especially at higher concentration.

The -OH radicals were generated from Fe3+/ascorbate/EDTA/H2O2 system by Fenton reaction which was scavenged by the extracts in a concentration dependent fashion where the competition between deoxyribose and extracts for hydroxyl radical generated from Fenton system. The scavenging effect of extracts against hydroxyl radicals was investigated by using the 2-deoxyribose oxidation method [16]. In the present study a significant correlation existed between the concentration and hydroxyl radical scavenging ability of the extract and was observed that AEDP has better scavenging activity than EEDP. Both the extracts were consumed in lesser concentrations than standard ascorbic acid.

In metal chelating assay, Ferrozine produces a violet complex with Fe2+. In the existence of a chelating agent, complex formation is broken up and as a result the violet color of the complex is decreased. Iron can stimulate lipid peroxidation by the Fenton response. Metal chelating potential is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation [17].

The results revealed that, the plant extracts are as good as ascorbic acid (AA) in which EEDP become extraordinary than AA.

Trypan blue is a stain used to identify dead tissue or cells. Dwelling cells or tissues with intact cellular membrane aren't colored, because the dye isn't always absorbed via the intact cell membrane. However it traverses the membrane of dead cells. Hence dead cells are shown as a distinctive blue color under the microscope [3]. In the trypan blue exclusion assay, there is a dose dependent inhibitory effect on both cancer cell lines treated with the extracts at increasing concentrations (5–250 mcg/ml) for 30 min. Incubation with extracts significantly affected the cytotoxic values which were maximum at the concentration of 250 mcg/ml. The % of cytotoxicity (dead cells) was in the following order: EEDP against DAL (93%), EEDP against EAC (94%), AEDP against DAL (75%) and AEDP against EAC (71%).

Lactate dehydrogenase (LDH) is an oxidative enzyme that changes lactate into pyruvate during glycolysis. It is present in cell membranes and cytoplasm. Intracellular LDH leakage is a well known indicator of cell membrane integrity and cell viability. The leakage of LDH from the injured cells was measured which is a more reliable and accurate marker of cytotoxicity [18]. The LDH leakage from DAL and EAC cell lines may be due to the cytotoxic nature of the plant extracts EEDP and AEDP. The order of % cytotoxicity was in the following order: EEDP and AEDP against DAL (94%), EEDP and AEDP against EAC (98%).

In total flavonoid content determination, aluminum chloride reacts with carbonyl group at C4 and hydroxyl at C3 (flavonols) and C5 in flavonols and flavones to form acid stable as well as unstable complexes. Similarly an another research report revealed that, reaction of aluminium chloride with flavonoids, causes unsaturation between C2 and C3 in the C ring, resulting in maximum absorption at 415. This unsaturated form may responsible for the improved antioxidant activity of flavones and flavonols [19].

COMPETING INTERESTS

We declare that we have no competing interests.

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