Evaluation of antioxidant and anti-tumour activity of *Pandanus Odorattissimus* Linn

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**ABSTRACT**

The antioxidant activity of MEPO has been studied by scavenging DPPH, Nitric acid, Superoxide radicals and hydroxyl radicals. The MEPO shows antioxidant activity by 87.52% reduced in DPPH and 73.55% inhibition of nitric acid. The result also indicates maximum inhibition of superoxide radical’s inhibition 74.12 and 78.14 % inhibition of hydroxyl radicals. The BHT was used as standard. In vitro cytotoxicity assay of MEPO was evaluated by using Trypan bluemethod. Determination of in vivo anti-tumor activity was performed after 24 h of EAC cells (2 X 106 cells/mouse) inoculation; MEPO (50 and 100 mg/kg i.p.) was administered daily for nine consecutive days. On day 10, half of the mice were sacrificed and the rest were kept alive for assessment of increase in life span. Anti-tumor effect of MEPO was assessed by the study of tumor volume, tumor weight, viable and non-viable cell count, hematological parameters. MEPO shows cytotoxicity (IC50 89.07±8.07 mg/mL) on EAC cell line. MEPO exhibited significant (p<0.01) decrease in tumor volume, tumor weight, viable cell count and percentage increased the life span (51.01, 58.04 and 87.09%) of EAC-treated mice. Hematological profile, tissue antioxidant assay significantly (p<0.01) reverted to normal level in MEPO-treated mice. MEPO has shown potent anti-tumor activity that may be due to its direct cytotoxic activity or antioxidant properties.

**Key words:** Pandanus odorattissimus, anti-tumor, antioxidant, mice.

**INTRODUCTION**

Oxidative stress results from an imbalance between the generations of oxygen derived radicals and the organism’s antioxidant potential playing an important role in many chronic diseases (Abdollahi et al., 2004). There is increasing evidence to show the involvements of free radicals and reactive oxygen species in a variety of disease, they can cause damage to cellular biomolecules such as nucleic acid, protein, lipids and carbohydrates and consequently may adversely affect immune function (Sheetal et al., 2008). The efficacy of a plant extract as an antioxidant is best evaluated based on results obtained by commonly accepted assays taking into account different oxidative conditions, system compositions and antioxidant mechanism (Prior and Wu, 2005). It is believed that medicinal plants are a potential source of antioxidant and ROS scavenger molecules (Arora et al., 2005). Natural antioxidants tend to be safer and they also possess antiviral, anti-tumor, hepatoprotective properties (Lim and Murtijaya, 2007). Cancer results from a single cell beginning to divide uncontrollably and forming a tumor and these cells differ from normal cells in that they are no longer responsive to normal growth controlling mechanisms (Bertram, 2001). The cytotoxic activities of plant extracts can be tested in various cell lines using cell viability assays such as MTT and Trypan blue among others. Major drawbacks of the current chemo-preventive practices (chemotherapy and radiation therapy) are the side effects and suppression of the immune system (Devasagayam and Sainis, 2002). Plants contain abundant quantities of less toxic and more effective substances. Therefore, efforts are being made to identify naturally occurring anticarcinogens which would prevent, slow and/or reverse the cancer induction and development. Apoptosis is a specific process that leads to programmed cell death which is essential in maintaining the stability of normal body tissues and occurs in various physiological and pathological situations (Hengartner, 2000). Pandan is said to be a restorative, deodorant, indolent and phylactic, promoting a feeling of well-being and acting as a counter to tropical latitude. It may be chewed as a counter to tropical latitude. It may be chewed as a breath sweetener or used as a preservative on foods. It is also said to have healthful properties, including antiviral, anti-allergy, antiplatelet,
anti-inflammatory, antioxidant and anti-tumor. Ayurvedic science finds the medicinal action of the essential oil yielded by the screw pine's highly scented flowers to be useful in headaches, ear aches and as a liniment for rheumatic pains. The distilled water made from the flowers is used for inducing perspiration. It is also prescribed as a stimulant and an antispasmodic. The flowers themselves are powdered and included in medicines, which are either sniffed like snuff or smoked for asthma and other bronchial infections (Keerthikar and Basu, 2000). Hence, the present investigation to study antioxidant property of *Pandanus odorattissimus* is undertaken.

**MATERIALS AND METHODS**

Aerial leaf parts of *Pandanus. Odorattissimus* Linn sps were collected from Gurmitkal, near Gulbarga, north Karnataka, India. The botanical identification was made by Dr. Shiddamallya N, Scientist, National Ayurveda Dietetics Research Institute (NADRI), Bangalore. A voucher specimen was deposited in department (RRCBI- 12749).

**Preparation of extract**

Twenty five gram of the powders of *Pandanus odorattissimus* leaf and its components was extracted in Methanol (250 mL) at respective boiling point of extracts. The extract was filtered using Whatman 1 filter paper, pooled and concentrated to dryness under reduced pressure.

**Chemicals**

Ethylene Diamine Tetra Acetate (EDTA) and Folin Ciocalteu’s reagent were purchased from SD fine chemicals, Mumbai, India. 1, 1-Diphenyl-2-picryl hydrazyl (DPPH), riboflavin, Nitro Blue Tetrazolium (NBT) chloride and pyrogallol were purchased from Himedia Lts, India. Potassium ferricyanide was purchased from Qualigens Fine Chemicals, India. Trichloroacetic acid (TCA) and Iron (II) chloride (FeCl₃) from E. Merck India Ltd, Indigo Caramine was purchased from SD Fine Chemicals, India.

**Free radical scavenging activity**

A stock solution of DPPH (1.3 mg mL⁻¹ in methanol) was prepared such that 75µl of it in 3 mL methanol gave an initial absorbance of 0.9. Decrease in the absorbance in the presence of sample extract at different concentration was noted after 10min. EC₅₀ was calculated for percentage of inhibition. A 0.1mM solution of DPPH in methanol was prepared and 1.0 mL of this solution was added to 3.0mL of control standard Butylated Hydroxyl Toluene (BHT) at different concentration (25-100µg mL⁻¹) and test solution at different concentration (5-100µg mL⁻¹) in different test tubes. The absorbance was measured at 517 nm.

**Nitric oxide scavenging activity**

Nitric oxide scavenging activity was measured by the spectrophotometer method (Madan et al., 2005). Sodium nitroprusside (5 mmol) in phosphate-buffered saline was mixed with a control without the test compound, but with an equivalent amount of methanol. Test solutions at different concentration (5-100µg mL⁻¹) were dissolved in methanol and incubated at 25°C for 30min. After incubation, 1.5 mL of incubated solution was removed and diluted with 1.5 mL of Griess reagent (1% naphthylethlenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of the nitrite with sulphanilamide and the subsequent coupling with naphthylethylene diamine dihydrochloride was measured at 546 nm.

**Superoxide radical scavenging activity**

Assay for superoxide radical scavenging the activity was based on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system (Ravi Shankar et al., 2002). The reaction mixture contained 50nm phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA, NBT 0.1 mg/3 mL, added in that sequence. The reaction was started by illuminating the reaction mixture with different concentrations of sample extract for 150 sec. Immediately after illumination, the absorbance was measure at 590 nm and EC₅₀ was calculated. Methanol was used for blank reading.

**Hydroxyl radical scavenging activity**

The scavenging capacity for hydroxyl radical was measured according to the modified method (Rajeshwar et al., 2005). The assay was performed by adding 0.1 mL EDTA, 0.01 mL of FeCl₃, 0.01 mL of H₂O₂, 0.36 mL of deoxyriboase, 1.0 mL of test solution (5-100 µg mL⁻¹) dissolved in distilled water, 0.33 mL of phosphate buffer (50 mM, pH 7.4) and 0.1 mL of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1 h a 1.0 mL portion of the incubated mixture was mixed with 1.0 mL of 10% TCA and 1.0 mL 0.5% TBA to develop the pink chromogen. The optical density was measured at 532 nm.

**Statistical analysis**

The results are presented as Mean±SEM. All the parameters
were analyzed by using student’s t-test. The data was considered as significant, if the p-value is p<0.05.

ANTITUMOUR ACTIVITY

Chemicals

Chemicals used were Trypan blue, 5-fluorouracil (Merck Limited, Mumbai, India), trichloroacetic acid, thiobarbituric acid, phenazinemethosulphate, reduced nicotinamide adenine dinucleotide, nitrobluetetrazolin and dithionitrobenzene. They were obtained from Sigma-Aldrich, Bangalore, India. All the chemicals and reagents were used in highest analytical grade.

Animals

Male Swiss albino mice (20–25 g) of 8 weeks of age were used for the experiment. The mice were grouped and housed in polyacrylic cages (38, 23 and 10 cm) with not more than six animals per cage. The animals were maintained under standard laboratory conditions (temperature 25–30°C and 55–60% relative humidity with dark/light cycle 14/10 h) and were allowed free access to standard dry pellet diet and water ad libitum. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All the procedures described were reviewed and approved by the University.

Acute toxicity

MEPO was administered orally to male Swiss albino mice to evaluate the acute toxicity as per the reported method (Lorke, 1983).

Transplantation of tumor cell

EAC cells were obtained from National Center for Cell Science, Pune, (NCCS Pune, India). The EAC cells were maintained in vivo in Swiss albino mice by intraperitoneal transplantation of 2 X 10^6 cells per mouse after every 10 days and it is used for both in vivo and in vitro study (Haldar et al., 2010).

Assay for in vitro cytotoxicity

In vitro short term cytotoxicity of MEPO was assayed by using Trypan blue exclusion method (Dolai et al., 2012). At first, we have prepared different concentration of MEPO solution as 25, 50 and 100 mg/mL. Then, 1X 10^6 EAC cells were suspended in 0.1 mL of phosphate buffered saline (PBS, 0.2 M, pH 7.4) and mixed with 100 mL of various afore mentioned concentrations of MEPO. Final concentration (25, 50 and 100mg/mL) of MEPO was adjusted by PBS and incubated at 37 °C for 3 h. After the completion of incubation, the viability of the cells was determined using Trypan blue dye (0.4% in normal saline) and the percentage of cytotoxicity was determined by calculating % inhibition and IC50 values.

Treatment schedule for assessment of in vivo antitumor potential

Swiss albino mice (20–25 g) were divided into five groups (n 12). All the animals in each groups except Group-I received EAC cells [2 X 10^6 cells/mouse intraperitoneally (i.p.)]. This was taken as day “0”. Group-I served as normal saline control (5 mL/kg i.p.). Group-II served as EAC control. After 24h of EAC transplantation, Groups-III, IV and V received MEPO at a dose of 50 and 100 mg/kg i.p. for nine consecutive days, respectively. Group-VI received reference drug 5-FU (20 mg/kg i.p.) for nine consecutive days (Bala et al., 2010). After administration of the last dose, six mice from each group were kept fasting for 18 h and blood was subsequently collected by direct cardiac puncture for the estimation of hematological and determination of serum biochemical parameters. The animals were then sacrificed for collection of liver and kidney tissues to check the different antioxidant parameters. The rest of the animals, in each group were kept alive and given food and water ad libitum to evaluate percentage increase in their life span to determine the mean survival time (MST). Anti-tumor activity of MEPO was assessed by observation of changes with respect to the following (tumor volume, tumor weight, percentage increase in life span (%ILS) viable/ non-viable tumor cell count, hematological parameters, biochemical parameters and tissue antioxidant assay) parameters.

Determination of tumor volume and tumor weight

The ascites fluid was collected from the peritoneal cavity. The volume was measured in a graduated centrifuge tube and expressed in milliliter (mL). The tumor weight was measured by weighing the mice before and after the collection of the ascetic fluid from peritoneal cavity and expressed in grams (g).

Percentage increase in life span

The effect of MEPO on percentage increased in life span was calculated on the basis of the mortality rates of the
Figure 1: Effect of MEPO compared with standard BHT for Inhibition of DPPH. Values are Mean±SEM. 6 independent analysis **p<0.001.

experimental mice, expressed as:

MST in days = (First death + Last death) / 2

ILS% = ((MST of the treated group − MST of the control group) / MST of the control group) × 100:

**Estimation of viable/non-viable tumor cell count**

The ascites fluid was taken in a white blood cell (WBC) pipette and diluted to 20 times with PBS. A drop of the diluted cell suspension was then placed on the Neubauer's counting chamber and the number of cells counted. The viability and non-viability of the cells were checked by Trypan blue dye exclusion assay method. The cells were stained with Trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the dye were nonviable. The viable and nonviable cells were determined by using the following formula:

Cell count = (Number of cells × dilution factor) / (Area × thickness of liquid film).

**Determination of hematological parameters**

Collected blood was used for the estimation of hemoglobin (Hb), red blood cell (RBC) and WBC count by standard procedures (D'Armour et al., 1965; Wintrobe et al., 1961).

**Statistical analysis**

All the experimental data are expressed as the mean SEM. Data was statistically analyzed by using one way analysis of variance followed by Dunnett’s post hoc test by Instat software (version 4). pValues of 50.01 were considered statistically significant.

**RESULTS**

**Inhibition of DPPH radicals**

The potential decrease in the concentration of DPPH radicals is due to the scavenging ability of MEPO and BHT (standard reference) showed the significant free radical scavenging activity 91.55 and 87.52% of inhibition, respectively, at 100µg mL⁻¹. The (IC₅₀) inhibitory concentration at which there is 55% reduction of free radical of MEPO was found to be 36 µg/mL⁻¹ (Figure 1).

**Nitric oxide scavenging activity**

The scavenging activity of nitric oxide by MEPO and BHT was mainly concentration depend on various concentrations of the extract. There was a moderate inhibition of nitric oxide formation, with the maximum inhibition 73.50 and 81.35% at 100 µg mL⁻¹ of Pandanus odorattissimus and BHT was observed (Figure 2).

**Superoxide radicals scavenging**

A moderate inhibition of the superoxide radicals was observed with 100 µg mL⁻¹ each of MEPO and BHT 74.12 and 79.67%, respectively (Figure 3).

**Hydroxyl radical activity**

The MEPO and BHT have shown a significant inhibition on hydroxyl radical and iron (II)- depend deoxyribose damage at all concentrations. The percentage of inhibition of hydroxyl radical being 78.17% and BHT 74.30%, respectively at 100 µg mL⁻¹ (Figure 4).
The MEPO was found to be safe in male Swiss albino mice up to the dose of 3000 mg/kg body weight peroral.

**Acute toxicity**

The MEPO was found to be safe in male Swiss albino mice up to the dose of 3000 mg/kg body weight peroral.

**In vitro cytotoxicity**

From the in vitro cytotoxicity study, it is clear that MEPO showed direct cytotoxic effect on the EAC cell line, with an IC$_{50}$ value of 89.07 ± 8.07 mg/mL (Figure 5).

**Tumor growth and survival parameters**

Anti-tumor activity of MEPO against EAC tumor bearing mice was assessed by the parameters such as tumor volume, tumor weight, cell count (viable and non-viable) MST and %ILS. The tumor volume, tumor weight and viable cell count were found to be significantly ($p<0.01$) increased and non-viable cell count declined significantly ($p<0.01$) in EAC control animals, when compared with normal control animals (Table 1). Administration of MEPO at doses of 25, 50 and 100 mg/kg significantly ($p<0.01$) decreased the tumor volume and viable cell count. Non-viable cell count was significantly ($p<0.01$) higher in MEPO treated animals as compared to EAC control animals. Furthermore, the MST was increased to 29, 32 (% ILS 58.04) and 38.04(% ILS 87.09) on administration of MEPO 25, 50 and 100 mg/kg, respectively.

**Hematological Parameters**

There was significantly ($p<0.01$) elevated levels of WBC and a significant ($p<0.01$) reduction of RBC and Hb levels in EAC control group as compared to the normal control group (Table 2). Administration of MEPO at doses of 50 and 100 mg/kg significantly ($p<0.01$) reduced WBC count in respect to EAC control group. RBC count and Hb content were found to be significantly ($p<0.01$) restored to the normal levels. These results implied the protective effect of MEPO on the hematological profile of EAC bearing mice.

**DISCUSSION**

Oxidative stress, in large quantities of Reactive Oxygen Species (ROS) like hydrogen peroxide, superoxide(O$_2^-$), hydrogen radical (OH$^-$), singlet oxygen generated is one of the earliest responses to stress. These ROS have a role in disease and aging in animals (Halliwell and Gutteridge, 1998). The antioxidative system protects the organism against ROS induced oxidative damage. There are restrictions on the use of synthetic antioxidants, such as BHT, because they are suspected to be carcinogenic (Govindrajan et al., 2003). Therefore, natural antioxidants have gained importance. The DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm. Which is induced by antioxidants? DPPH radicals react with suitable reducing agents and then electrons become paired off and the solution loses color stoichiometrically with the number of electrons taken up (Blois, 1958). The significant decrease in the concentration of the DPPH radical is due to the scavenging ability of Pandanus odoratissimus. The nitric oxide was generated by sodium nitroprusside and measured by the Greiss reduction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to
Table 1: Effect of MEPO on tumor volume, tumor weight, total cell count, viable and nonviable cell count, MST and ±%ILS in EAC bearing mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>EAC MEPO (25 mg/kg)</th>
<th>EAC MEPO (50 mg/kg)</th>
<th>EAC MEPO (100 mg/kg)</th>
<th>EAC 5-FU (20 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor volume (mL)</td>
<td>2.75 ± 0.13</td>
<td>1.66 ± 0.28</td>
<td>1.40 ± 0.13*</td>
<td>0.91 ± 0.05*</td>
<td>0.53 ± 0.05*</td>
</tr>
<tr>
<td>Tumor weight (g)</td>
<td>3.21 ± 0.10</td>
<td>1.20 ± 1.02</td>
<td>1.08 ± 0.08*</td>
<td>0.83 ± 0.06*</td>
<td>0.57 ± 0.04*</td>
</tr>
<tr>
<td>Total cell (10^7 cell/mL)</td>
<td>9.30 ± 0.43</td>
<td>5.00 ± 0.13</td>
<td>4.00 ± 0.13*</td>
<td>3.89 ± 0.05*</td>
<td>3.81 ± 0.09*</td>
</tr>
<tr>
<td>Viable cell (10^7 cell/mL)</td>
<td>8.92 ± 0.42</td>
<td>3.15 ± 1.18</td>
<td>2.78 ± 0.18*</td>
<td>0.94 ± 0.08*</td>
<td>0.60 ± 0.08*</td>
</tr>
<tr>
<td>Nonviable cell (10^7 cell/mL)</td>
<td>0.38 ± 0.04</td>
<td>1.45 ± 0.17</td>
<td>1.22 ± 0.07*</td>
<td>2.96 ± 0.08*</td>
<td>3.21 ± 0.09*</td>
</tr>
<tr>
<td>Viable %</td>
<td>95.91</td>
<td>71.25</td>
<td>69.50</td>
<td>24.16</td>
<td>15.75</td>
</tr>
<tr>
<td>Nonviable %</td>
<td>4.09</td>
<td>28.25</td>
<td>30.50</td>
<td>76.09</td>
<td>84.25</td>
</tr>
<tr>
<td>MST (days)</td>
<td>21</td>
<td>29</td>
<td>32</td>
<td>38</td>
<td>43</td>
</tr>
<tr>
<td>±%ILS</td>
<td>00</td>
<td>51.01</td>
<td>58.04</td>
<td>87.90</td>
<td>104.76</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM, where n=6. *p<0.01 for treated groups versus EAC control group. ±

Table 2: Effect of MEPO on hematological parameters in EAC bearing mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>EAC MEPO (25 mg/kg)</th>
<th>EAC MEPO (50 mg/kg)</th>
<th>EAC MEPO (100 mg/kg)</th>
<th>EAC 5-FU (20 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (cell_10^6/mL)</td>
<td>5.89 ± 0.20</td>
<td>3.25 ± 1.02</td>
<td>4.81 ± 0.23b,*</td>
<td>5.09 ± 0.70b,*</td>
<td>5.19 ± 0.20b,*</td>
</tr>
<tr>
<td>WBC (cell_10^3/mL)</td>
<td>5.16 ± 0.32</td>
<td>6.13 ± 0.02</td>
<td>7.53 ± 0.36b,*</td>
<td>6.16 ± 0.27b,*</td>
<td>5.09 ± 0.33b,*</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>11.48 ± 0.98</td>
<td>7.00 ± 0.30</td>
<td>7.98 ± 0.41b,*</td>
<td>9.86 ± 0.41b,*</td>
<td>10.35 ± 1.16b,*</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM, where n=6.

produces nitrates ions that can be estimated by using Greiss reagent. Scavengers of nitric oxide compete with the oxygen reduced production of nitric oxide. There was a moderate inhibition of the superoxide radical, with the maximum inhibition being 66% at 1 mg mL⁻¹ extract concentration. Superoxide anion is oxygen centered radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reaction and by non-enzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complex...
such as cytochrome (Gulcin et al., 2003).

The potentially reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins. The effect of *Pandanus odorattissimus* on the inhibition of free radical mediated deoxyribose damage was assessed by means of iron (II) dependent DNA damage assay, which showed significant results (Jornot et al., 1998). The Ehrlich ascites tumor implantation induces a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration, and a progressive ascites fluid accumulation (Bala et al., 2010). The ascites fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells. The present study showed that MEPO significantly decreased the tumor volume, tumor weight, viable tumor cell and increased the non-viable tumor cell and life span of the EAC bearing control mice. The reliable criteria for judging the value of any anticancer drug are prolongation of life span. The observed increase in the lifespan of tumor bearing mice by reduction of nutritional fluid volume and seization of the tumor growth is a positive result and further corroborates the anti-tumor potential of MEPO. Usually in cancer chemotherapy, the major problems are myelo suppression and anemia. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or Hb percentage, and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions (Haldar et al., 2011). Pharmacotherapy with MEPO replenishes the RBC and Hb content to the normal levels. The WBC count was reduced as compared with that of EAC control mice. These indicating parameters revealed that MEPO extracted less toxic effect to the hemopoietic system and plausibly had selective affinity to the tumor cell and thereby it could maintain the normal hematological profile. It is evident from the result that MEPO possesses a protective action on hemopoietic system.

**CONCLUSION**

*Pandanus odorattissimus* leaf extract demonstrated moderate activity of antioxidant, reducing power and scavenging activity. Purification of the extract may lead to increase activity in its bioactive compounds. The antioxidants activity of MEPO may be due to its proton donating capability as shown in the DPPH radical scavenging results. Acting as an electron donor that can react with free radicals it converts them to more stable products and terminate radical chain reactions. Findings suggest that the MEPO exhibits potential anti-tumor and antioxidant activities which enlighten a novel source of phytomedicines in free radical and tumor biology. The potential anti-tumor activity of MEPO is presumably potentiated by its direct cytotoxic effect and antioxidant property. It was assumed that attenuation of oxidative stress in different tissues in EAC bearing mice decreased the viability of EAC cells. Finally, our findings suggest that the MEPO exhibits potential anti-tumor and antioxidant activities which enlighten a novel source of phytomedicines in free radical and tumor biology.

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