Apopogossypolone, derivative of gossypol, mobilizes endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage

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ARTICLE INFO

Article history:
Received 13 December 2011
Accepted 14 April 2012
Available online 24 April 2012

Keywords:
Gossypol
Apopogossypolone
Endogenous copper
Oxidative DNA breakage
Anticancer mechanism

ABSTRACT

Gossypol is a polyphenolic aldehyde that is produced in the cotton plant. Since long it has been reported to possess antiproliferative activity against a variety of cancer cell lines as well as tumor regression in animal models. However, the toxicity of gossypol does not permit it to be an effective antitumor agent. One of the derivatives of gossypol to show promising results is apogossypolone. For example, it has been shown to specifically target tumor growth in hepatocellular carcinoma xenograft in nude mice without causing any damage to normal tissue. Using human peripheral lymphocytes, in this paper we show that both gossypol and its semi-synthetic derivative apogossypolone cause oxidative DNA breakage in these cells through the mobilization of endogenous copper ions. Such cellular DNA breakage is inhibited by copper specific chelator but nor by iron or zinc chelating agents. Similar results are obtained with isolated nuclei indicating that chromatin bound copper is mobilized in this reaction. Further, apogossypolone showed enhanced DNA breakage and increased oxidative stress in whole lymphocytes as compared with gossypol indicating that this is possibly the result of greater permeability of apogossypolone. It is well established that tissue, cellular and serum copper levels are considerably elevated in various malignancies. Therefore, cancer cells may be subject to greater electron transfer between copper ions and gossypol/apogossypolone to generate reactive oxygen species responsible for DNA cleavage. This may account for the preferential cytotoxicity of apogossypolone towards tumor cells.

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1. Introduction

Gossypol [1,1',6',6'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2'-binaphthalene)-8,8'-dicarboxaldehyde], a polyphenolic aldehyde produced in the roots, leaves, stems and seeds of the cotton plant (Fig. 1). Gossypol has been reported to have antiproliferative activity on a variety of cancer cell lines including breast (Ligueros et al., 1997), bladder (Macoska et al., 2008), pancreas (Mohammad et al., 2005), lung (Kilic et al., 2009), colon (Zhang et al., 2009), prostate (Huang et al., 2008), and head and neck (Bauer et al., 2005; Wolter et al., 2006). It also inhibits proliferation of Dunning prostate cancer cells and reduces metastasis of these cells in xenograft Copenhagen rat models (Jiang et al., 2000). It has been shown that gossypol induces apoptosis and DNA fragmentation in DU-145 cells (Huang et al., 2006). However, the toxicity of gossypol does not permit it to be an effective antitumor agent. Nevertheless, gossypol represents a potentially interesting lead compound (Kitada et al., 2003; Zhang et al., 2009) for the synthesis of novel anticancer agents. One of the derivatives of gossypol to show promising results is apogossypolone (ApoG2) (Arnold et al., 2008). It is assumed to have reduced non-specific reactivity, thereby decreasing toxicity of gossypol. The main modification is the removal of the aldehyde groups of gossypol. Further, four quinonoid moieties have been added resulting in enhanced permeability of the compound.

ApoG2 has been shown to specifically target tumor growth in hepatocellular carcinoma xenograft in nude mice without causing damage to normal tissue (Mi et al., 2008). Further, ApoG2 has also been shown to induce apoptosis in follicular Small Cleaved Cell Lymphoma model, pre-B-acute lymphoblastic leukemia, marginal zone lymphoma and chronic lymphocytic leukemia (Arnold et al., 2008). It has also been shown to induce apoptosis and suppress tumor growth in nasopharyngeal carcinoma xenografts (Hu et al., 2008). The primary mechanism by which these compounds inhibit proliferation and induce apoptosis in cancer cells, sparing normal tissues from toxicity is unknown.
cells, is not known and has been the subject of considerable interest (Mehrpour et al., 2009).

Previous studies in our laboratory have centered on the mechanisms of anticancer properties of plant derived polyphenolic compounds. We have shown that various classes of plant polyphenols are able to cause oxidative breakage of cellular DNA either alone or in the presence of transition metal ions such as copper (Azmi et al., 2005; Bhat et al., 2007; Hanif et al., 2008; Khan et al., 2011). We have hypothesized that the cytotoxic action of these compounds against cancer cells involves mobilization of endogenous copper and consequent prooxidant action (Hadi et al., 2000, 2007). Towards validation of our hypothesis considerable evidence has been deduced over the years (Shamim et al., 2000, 2007). Towards validation of our hypothesis considerable evidence has been deduced over the years (Shamim et al., 2000, 2007). We have hypothesized that the cytotoxic action of these compounds against cancer cells involves mobilization of endogenous copper and consequent prooxidant action (Hadi et al., 2000, 2007). Towards validation of our hypothesis considerable evidence has been deduced over the years (Shamim et al., 2000, 2007). We have hypothesized that the cytotoxic action of these compounds against cancer cells involves mobilization of endogenous copper and consequent prooxidant action (Hadi et al., 2000, 2007). Towards validation of our hypothesis considerable evidence has been deduced over the years (Shamim et al., 2000, 2007). We have hypothesized that the cytotoxic action of these compounds against cancer cells involves mobilization of endogenous copper and consequent prooxidant action (Hadi et al., 2000, 2007).

2. Materials and methods

2.1. Materials

Gossypol, neocuproine, bathocuproine disulphonate, superoxide dismutase (SOD), agarose, low melting point agarose, RPMI 1640, Triton X-100, Trypan blue, Histopaque1077, and PBS (Ca²⁺ and Mg²⁺ free) were purchased from Sigma (St. Louis, MO). Other chemicals were of analytical grade. Fresh solutions of gossypol and ApoG2 were prepared as a stock of 3 mM in DMSO. In all studies (except ferrous oxidation-xylene orange (FOX) assay and TBARS estimation), the reaction mixture contained 2% DMSO (v/v). To test any effect of solvent on the studies, DMSO solution was added to the cells at the final concentration of 2% v/v, which was the highest concentration of DMSO used in the compound-treated reaction medium. No difference was observed with or without DMSO, indicating that the DMSO at the tested concentrations did not influence the results. For FOX assay and the estimation of TBARS in lymphocytes, the solvent control concentration varied between 1% and 12% v/v and again no influence of the solvent was observed on the results. On addition to reaction mixtures, in the presence of buffers mentioned and at concentrations used, the polyphenols used remained in the solution. The volumes of stock solution added did not lead to any appreciable change in the pH of reaction mixtures.

Lymphocytes were isolated from heparinized blood samples using Histopaque1077. Three milliliter blood was drawn from a single healthy non-smoking donor for the entire study on several occasions by venepuncture and diluted suitably in Ca²⁺ and Mg²⁺ free PBS. The cells were finally suspended in RPMI 1640.

2.2. Detection of Cu(I) production

The selective sequestering agent neocuproine was employed to detect reduction of Cu(II) to Cu(I) by recording the formation of neocuproine–Cu(I) complex which absorb maximally at 450 nm. The reaction mixture (3 ml) contained 3 mM Tris–HCl (pH 7.5), fixed concentration (50 μM) of Cu(II), 300-μM neocuproine and fixed concentrations (50 μM) of gossypol/ApoG2. The reaction was started by adding Cu(II) and the spectra were recorded immediately afterwards.

2.3. Assessment of DNA breakage in isolated whole lymphocytes and lymphocyte nuclei

Treatment of whole lymphocytes with gossypol and ApoG2, and the subsequent Comet assay was performed essentially as described earlier (Ullah et al., 2009; Khan et al., 2011). However, since the DNA breakage had to be compared with that in lymphocyte nuclei, the treatment of cells with gossypol and ApoG2 was done on slides rather than in microcentrifuge tubes. Further, the lysis of cells was carried out after the treatment. Briefly, approximately 10,000 lymphocytes were mixed with 75 μl prewarmed 2% LMPA in PBS and immediately applied to a frosted microscope slide previously layered with 75 μl of 1% standard agarose in PBS. The slides were allowed to gel for 10 min at 4°C. Each slide was then transferred to a rectangular dish (8 cm × 3 cm × 5 mm) that contained a reaction mixture of gossypol/ApoG2 and other additions as mentioned in various legends to figures and tables. The slides with the reaction mixture were incubated at 37°C. The slides were then washed twice by placing in 0.4 M phosphate buffer pH 7.5 for 5 min at room temperature. Lysis of cells was then performed by submerging the slides in a tank containing lysis solution in the absence of light for 1 h at 4°C. The use of a tank instead of a coplin jar allowed simultaneous processing of a number of slides. The lysis solution (pH 10) consisted of 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris and 1% Triton X-100 added just prior to use. After lysis, slides were transferred to another tank containing 0.4 M phosphate buffer (pH 7.5) for 10 min. DNA unwinding and expression of alkali-labile sites were done by leaving the slides in the high-pH electrophoresis buffer at 4°C for 30 min. Subsequently, the electrophoresis, neutralization and staining of the slides were carried out as described earlier (Azmi et al., 2005).

In the lysed version of comet assay, essentially all the steps were carried out as above with the exception that the cells were lysed prior to treatment with reaction mixture. The lysed version of the comet assay has been used to study the direct interaction of various agents with cell nuclei as it eliminates the effect of cell membrane as a barrier and the cytoplasmic environment (Mazzanti, 1998). Comet images were observed at 100× magnification with a fluorescence microscope (Olympus CX41) and COHU 4910 equipped with a 510- to 560-nm excitation and 590-nm barrier filters integrated CC camera. Fifty images were randomly selected.
from each sample, and their lengths (diameter of the nucleus plus migrated DNA) were measured on the screen as automatically generated by Komet 5.5 image analysis system of Kinetic Imaging, Liverpool, UK.

2.4. Viability assessment of lymphocytes

The lymphocytes were checked for their viability before the start and after the end of the reaction using Trypan Blue Exclusion Test. The viability of lymphocytes was found to be more than 94%.

2.5. Determination of TBARS generation in lymphocytes and H2O2 in the reaction medium

Thiobarbituric-acid-reactive substance (TBARS) was determined according to the method of Ramanathan et al. (1994). A cell suspension (1 × 10^5 cells/ml) was incubated with gossypol and ApoG2 (0–400 μM) at 37 °C for 1 h and then centrifuged at 1000 rpm. In some experiments, the cells were pre-incubated with fixed concentrations of neocuproine and thiourea. The cell pellet was washed twice with PBS (Ca2+ and Mg2+ free) and suspended in 0.1 N NaOH. The cell suspension (1.0 ml) was further treated with 10% TCA and 0.6 M TBA (thiobarbituric acid) in boiling water bath for 10 min. The absorbance was read at 532 nm.

H2O2 was determined in the incubation medium (RPMI 1640) by FOX (Ferrous ion Oxidation-Xylenol orange) assay as described by Long et al. (2000). The simplified reaction sequence involves the oxidation of ferrous (Fe2+) to ferric (Fe3+) ions by H2O2 with the subsequent binding of the Fe3+ ion to the ferric-sensitive dye xylene orange, yielding an orange to purple complex, which is measured at 560 nm. The reaction mixture contained gossypol or ApoG2 along with the incubation medium used in the treatment of lymphocytes. After incubation for 2 h at 37 °C, an aliquot of 200 μl was analyzed for H2O2 formation (Shamim et al., 2008).

2.6. Statistics

The statistical analysis was performed as described by Tice et al. (2000) and is expressed as ±SEM of three independent experiments. A student’s t-test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. p-values ≤0.05 were considered statistically significant.

3. Results

3.1. Reduction of Cu(II) to Cu(I) by gossypol and apogossypolone

To analyze the copper reducing capacity of these two compounds, in the experiment shown in Fig. 2 we used neocuproine as sequesters Cu(I) selectively forming a complex that absorbs maximally at 450 nm. Under the conditions employed in the experiment neither Cu(II) nor the compounds alone interfere with this maxima whereas, gossypol as well as ApoG2 react with Cu(II) to generate Cu(I) as evidenced by the peak appearing at 450 nm. The result shows that both the compounds are capable of reducing Cu(II) to Cu(I) to a significant degree.

3.2. Cleavage of supercoiled pBR322 plasmid DNA by gossypol and ApoG2

To assess the DNA cleavage efficiency of gossypol and its synthetic derivative ApoG2, we analyzed the cleavage of supercoiled plasmid DNA to other topological forms. Fig. 3 shows that even at 200 μM concentration, gossypol and ApoG2 alone do not cause any change in the structure of the plasmid. Further, at the tested concentration (50 μM) copper alone does not cause any DNA breakage. However, in the presence of copper and at relatively lower concentration (100 μM) of the compounds, both the compounds show conversion of supercoiled DNA to open circular form. However, despite the DNA cleavage similarity, the relative cytotoxicity of ApoG2 has been found to be considerably greater in prostate cancer cells (Zhan et al., 2009). Presumably the cytotoxic mechanism of ApoG2 involves additional mechanisms for its cytotoxic action such as enhanced cell permeability.

3.3. Comparison of lymphocyte DNA breakage by gossypol and ApoG2

Alkaline version of comet assay makes it possible to detect DNA single-strand breaks and alkali-labile sites in cellular DNA. In the present experiment, we tested increasing concentrations of gossypol and ApoG2 (20, 25 and 50 μM) for DNA breakage in peripheral human lymphocytes using alkaline comet assay with whole lymphocytes and lymphocyte nuclei. Table 1 shows that there is a dose dependent increase in DNA breakage in whole lymphocytes as well
as lymphocyte nuclei by both the compounds. However, ApoG2 causes a significantly greater degree of DNA breakage in whole lymphocytes. Further, the result also shows that such DNA breakage is expressively enhanced in presence of exogenously added copper in whole lymphocytes. We further tested the effect of gossypol and ApoG2 on lymphocyte nuclei at the concentrations given above. Since the treatment of cell nuclei eliminates the cell membrane and cytoplasmic barrier, the compounds are able to directly interact with the nucleus. Thus the degree of permeability is not a factor in compound-induced DNA breakage. It is known that the nuclear pore complex is permeable to small molecules (Mazzanti, 1998). The DNA damage induced in cell nuclei is a result of direct interaction of the compounds with the nucleus. Therefore, this treatment should result in a higher degree of DNA breakage as compared to whole cells. Thus as expected, an enhanced level of DNA breakage is seen in cell nuclei. Both the compounds induce approximately similar degree of DNA breakage, in the case of nuclei. Thus the enhanced DNA breakage seen in whole lymphocytes with ApoG2 is possibly the result of greater permeability of the gossypol derivative.

Table 2 gives the results of the experiment where the effect of various scavengers of ROS was studied. We used copper chelators (neocuproine and bathocuproine disulphonic acid), to study their effect on DNA breakage by both the compounds in whole lymphocytes as well as in lymphocyte nuclei. In whole lymphocytes, a clear inhibition was seen in presence of Cu(I) specific chelating agents, neocuproine (—) or bathocuproine disulphonic acid (----). Values reported are mean ± SEM of three independent experiments. "value was found to be significant when compared to control (no treatment) value and **value at P < 0.01. Similarly, "value was found to be significant when compared to control (no treatment) value and **value at P < 0.01.

![Fig. 4. Gossypol (50 μM, □) and ApoG2 (50 μM, △)-induced DNA degradation in whole lymphocyte cells (A) and in lymphocyte nuclei (B) in presence of Cu(I) specific chelating agents, neocuproine (——) or bathocuproine disulphonic acid (----). Values reported are mean ± SEM of three independent experiments. "value was found to be significant when compared to control (no treatment) value and **value at P < 0.01.](image)

Table 1
Comparison of DNA breakage in intact lymphocytes and lymphocyte nuclei by gossypol and ApoG2 as a function of comet tail length.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Whole lymphocytes</th>
<th>Lymphocyte nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gossypol</td>
<td>ApoG2</td>
</tr>
<tr>
<td></td>
<td>Alone</td>
<td>With 20 μM copper</td>
</tr>
<tr>
<td>0</td>
<td>3.04 ± 0.2</td>
<td>3.09 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>8.60 ± 0.6*</td>
<td>16.41 ± 1.02*</td>
</tr>
<tr>
<td>20</td>
<td>18.11 ± 1.09*</td>
<td>23.77 ± 1.61*</td>
</tr>
<tr>
<td>50</td>
<td>25.89 ± 1.77*</td>
<td>37.05 ± 2.03*</td>
</tr>
</tbody>
</table>

*Values were found to be significant when compared to gossypol alone at the same concentration at P < 0.05. **values were found to be significant when compared to ApoG2 alone at the same concentration at P < 0.05.

Cells were preincubated with scavengers of ROS and then treated with the compounds for 1 h in dark at 4 °C. "value was found to be significant when compared to control (no treatment) value and **value at P < 0.01. Similarly, "value was found to be significant when compared to control (no treatment) value and **value at P < 0.01.

The DNA damage induced in cell nuclei is a result of direct interaction of the compounds with the nucleus. Therefore, this treatment should result in a higher degree of DNA breakage as compared to whole cells. Thus as expected, an enhanced level of DNA breakage is seen in cell nuclei. Both the compounds induce approximately similar degree of DNA breakage, in the case of nuclei. Thus the enhanced DNA breakage seen in whole lymphocytes with ApoG2 is possibly the result of greater permeability of the gossypol derivative.
lymphocyte nuclei treated with gossypol and ApoG2, both neocuproine and bathocuproine disulphonic acid were found to inhibit DNA breakage (Fig. 4B). In this case both neocuproine and bathocuproine disulphonic acid are directly able to interact with nuclei. We also tested the effect of desferoxamine (iron specific chelator) and histidine (zinc specific chelator) on compound induced DNA breakage using whole lymphocytes as well as nuclei. However, both the chelators did not cause any inhibition of DNA breakage by gossypol or ApoG2. We take these results to suggest that gossypol and its derivative ApoG2 mobilize chromatin bound copper leading to oxidative DNA breakage Fig. 4.

3.4. Comparison of TBARS generation in lymphocytes by gossypol and ApoG2

Damage by hydroxyl radicals and other reactive oxygen species to deoxyribose gives rise to thiobarbituric acid (TBA) reactive substances (Smith et al., 1992; Quinlan and Gutteridge, 1987). Therefore, we have determined the formation of TBA reactive substances (TBARS) in lymphocytes as a measure of oxidative stress with increasing concentration of gossypol and ApoG2. The effect of preincubating cells with neocuproine and thiourea on the level of TBARS, was also tested for both the compounds. Fig. 5A and B shows that both the compounds give a dose-dependent increase in TBARS generation. As seen from the results, ApoG2 generates significantly higher levels of TBARS than gossypol. This may be attributed to greater cell permeability of ApoG2 and subsequently a higher degree of hydroxyl radical generation by ApoG2 in cells. Moreover, when the cells were preincubated with neocuproine and thiourea there was a considerable decrease in TBARS level by both the compounds. Thus, it can be suggested that both DNA breakage and oxidative stress in cells involves the interaction of gossypol and ApoG2 with intracellular copper and its reduction to Cu(I).

3.5. Generation of hydrogen peroxide in the incubation medium

Polyphenols have been shown to auto-oxidize in cell culture media and lead to the release of H$_2$O$_2$ and quinone that can enter the cells/nuclei causing damage to various biomacromolecules (Long et al., 2000). This extraneous production of ROS could also account for DNA breakage observed above. Therefore, we determined the production of H$_2$O$_2$ by gossypol and ApoG2 in...
the incubation medium RPMI 1640 and compared it to a known producer, tannic acid. As can be seen in Fig. 6, where the generation of H₂O₂ is notably significant by tannic acid, gossypol and ApoG2 do not produce H₂O₂ in any significant amount. This indicates that the DNA breakage by the compounds observed above is not a result of extraneous generation of H₂O₂ in the reaction medium. Moreover, we have earlier shown that there exists no correlation between the extraneous generation of H₂O₂ and the ability of polyphenols to cause oxidative DNA breakage (Ullah et al., 2009).

4. Discussion

The above results lead to the following conclusions: (1) the cellular DNA breakage in normal lymphocytes by gossypol and ApoG2 involves redox-cycling of endogenous copper, possibly chromatin bound copper; (2) the relatively higher DNA breakage efficiency of ApoG2 is possibly accounted for by the presence of quinonoid moieties which impart a greater degree of membrane permeability. Copper mediated further oxidation of ortho-hydroxyl groups in ApoG2 may lead to an even greater degree of DNA breakage capacity. As already mentioned both gossypol and ApoG2 have been shown to possess anticancer and apoptosis inducing properties in various cancer cell lines (Huang et al., 2006; Zhang et al., 2009; Mi et al., 2008; Arnold et al., 2008). Thus gossypol and ApoG2 fall under the category of plant polyphenols such as flavonoids and catechins (considered to possess anticancer properties) that are able to mobilize endogenous copper leading to the formation of reactive oxygen species and consequent cellular DNA breakage (Hadi et al., 2000, 2007; Hanif et al., 2008; Shamim et al., 2008; Ullah et al., 2009; Khan et al., 2011). We believe that this could be an important mechanism for the anticancer properties of gossypol and ApoG2. We give below several lines of evidence in support of the above mechanism:

1. Copper is an important metal ion present in the chromatin and is closely associated with DNA bases particularly guanine (Bryan, 1979; Kagawa et al., 1994). Copper transporters are over expressed in malignant cells, which aid the uptake and accumulation of copper (Peng et al., 2006). It is well established that tissue, cellular and serum copper levels are considerably elevated in various malignancies (Gupte and Mumper, 2008). Therefore, cancer cells may be subject to greater electron transfer between copper ions and gossypol/ApoG2 to generate ROS. There are a number of studies which have focused on determining the concentrations of four important elements; copper, zinc, iron and selenium in patients with cancer (Kuo et al., 2002; Zuo et al., 2006). These studies showed that while zinc, iron and selenium concentrations were significantly lower in patients with cancer, the copper concentrations were almost always found to be significantly elevated (up to two- to threefold) compared to age-matched samples from normal tissue.

2. Fe²⁺ and Cu²⁺ are the most redox active of the metal ions in living cells. Wolfe et al. (1994) have proposed that a copper mediated Fenton reaction, generating site-specific hydroxyl radicals, is capable of inducing apoptosis in thymocytes. In a study with thiol-containing compounds, apoptosis was induced in different cell lines when either free copper or ceruloplasmin (a copper binding protein) was added; such activity was not observed, however, when either free iron or the iron-containing serum protein transferrin was added (Held et al., 1996).

3. Among oxygen radicals, the hydroxyl radical is most electrophilic with high reactivity and therefore possesses a small diffusion radius. Thus, in order to cleave DNA, it must be produced in the vicinity of cellular DNA (Pryor, 1988). The location of the redox-active metal is of utmost importance because the hydroxyl radical, due to its extreme reactivity, interacts exclusively in the vicinity of the bound metal. This is also in concurrence with the observation that gossypol induces apoptosis-like DNA fragmentation in HL-60 cells (Jarvis et al., 1994). Such intracellular DNA laddering often used as an indicator of apoptosis may reflect DNA fragmentation by non-enzymatic processes by metal-chelating agents that promote the redox activity of endogenous copper ions and the resulting production of hydroxyl radicals (Burkitt et al., 1996).

4. In normal cells, there exists a balance between the free radical generation and the antioxidant defense (Devi et al., 2000). However, it has been clearly documented that tumor cells are under persistent oxidative stress and have an altered antioxidant system (Schumacker, 2006) and thus further ROS stress in these malignant cells reaching a threshold level could result in apoptosis (Gupte and Mumper, 2008). These observations further suggest that neoplastic cells may be more vulnerable to oxidative stress because they function with a heightened basal level of ROS due to increased rate of growth and metabolism (Kong et al., 2000). Thus, in cancer cells, an enhanced exposure to ROS, generated through the redox activity of endogenous copper, can overwhelm the cells antioxidant capacity, leading to irreversible damage and cell death.

5. Conclusion

The significance of the results presented above lies not so much in the use of gossypol or its derivatives as antitumor agents but as in further confirmation of our hypothesis which envisages the mobilization of endogenous copper and the consequent generation of ROS. Since tumor cells are already under considerable oxidative stress any further increase in ROS levels is likely to be cytotoxic (Schumacker, 2006). Thus, the mechanism proposed by us would be an alternative, non-enzymatic and copper dependent pathway for the cytotoxic action of certain anticancer agents that are capable of mobilizing and reducing endogenous copper. Such a common mechanism better explains the anticancer effects of polyphenols with diverse chemical structures as also the preferential cytotoxicity towards cancer cells. Further, this also leads to the prospect of synthesizing novel anticancer compounds with greater permeability and half-life in cells and more efficient copper-chelation and -reducing capabilities.

Acknowledgments

The authors acknowledge the financial assistance Provided by the University Grants Commission, New Delhi, Under the BSR Programme. The authors also acknowledge the financial support of CSIR, New Delhi.

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