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Comparative nuclease and anti-cancer properties of the naturally occurring malabaricones

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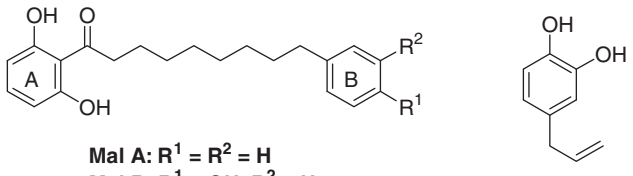
ABSTRACT

The nuclease activities of the malabaricones have been studied so as to establish a structure–activity correlation and deduce the mechanistic pathway of the process. The inactivity of malabaricone A and malabaricone D revealed that the resorcinol moiety, present in the malabaricones did not contribute to the nuclease activity. Amongst the test compounds, malabaricone C (mal C) containing a B-ring catechol moiety showed significantly better Cu(II)-dependent nuclease activity than the partially methylated catechol derivative, mal B and curcumin. Mal C was found to bind efficiently with Cu(II) and DNA to facilitate the DNA nicking via a site-specifically generated Cu(I)-peroxo complex. Consistent with its Cu(II)-dependent nuclease property, mal C showed better cytotoxicity (IC_{50} = 5.26 ± 1.20 μM) than curcumin (IC_{50} = 24.46 ± 3.32 μM) against the MCF-7 human breast cancer cell line. The mal C-induced killing of the MCF-7 cells followed an apoptotic pathway involving oxidative damage to the cellular DNA.

1. Introduction

There is a burgeoning interest in small organic molecules, capable of switching their redox status as these can often cleave DNA, and hence can be used as anti-cancer agents. Although, the redox potentials of a variety of metal ions have been exploited for the development of DNA cleaving agents, the organic compounds possibly play more important roles in this regard, as they provide multitude binding interactions with the target DNA, while ensuring the required electron transfer via their intrinsic chemical, electrochemical and photochemical properties.[1a,b] Several plant-derived polyphenolic antioxidants such as flavonoids,[2a] tannins,[2b] stillbenoids,[2c] and curcumins[2d] are capable of inducing oxidative DNA damage in the presence of certain transition metal ions especially Cu(II) via Fenton-mediated generation of the reactive active species (ROS). The mode of DNA binding and degradation by some of these compounds are similar to those of the known anti-cancer drugs such as bleomycin, adriamycin and 40-(9-acrdinylamino) methanesulphone-m-anisidine (mAMSA).[3a–c]

The fruit rind of the plant *Myristica malabarica* (Myristicaceae) (popularly known as rampatri, Bombay mace, or false nutmeg) is used as an exotic spice in various Indian cuisines. It is credited with hepatoprotective, anticarcinogenic and antithrombotic properties, and is found as a constituent in many Ayurvedic preparations such as pasupasi.[4a,b] Earlier, we have isolated four diarylnonanoids, malabaricones A–D (designated as mal A–D) from its methanol extract and found that two of these compounds, mal B and mal C, especially the latter possess superior in vitro antioxidant activity than curcumin.[5a] More recently, mal B and mal C were found to accelerate healing of indomethacin-mediated gastric ulcer healing in mice via their antioxidant action.[5b,c] The chemical structures of the malabaricones are shown in Figure 1. It is well-known that many antioxidants can perturb the cellular redox conditions specifically in cancer cells, leading to elevated levels of ROS and subsequent cell death.[6] Hence, the primary aim of the study was to assess the potential of the malabaricones as the DNA cleaving agents, and whether this confer them with anti-cancer property. Our results revealed that amongst the test compounds, mal C and to some extent mal B could induce Cu(II)-mediated DNA nicking. Consistent with this result, mal C also showed maximum toxicity to the



Mal A: $R^1 = R^2 = H$
Mal B: $R^1 = OH$, $R^2 = H$
Mal C: $R^1 = R^2 = OH$
Mal D: $R^1 = R^2 = O\text{-}CH_2\text{-}O$

Figure 1. Chemical structures of the malabaricones and allyl pyrrocatechol.

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human breast cancer MCF-7 cells. The cytotoxicity of mal C followed an apoptotic pathway and involved oxidative DNA damage of the MCF-7 cells.

2. Results

2.1. Metal dependent DNA nicking abilities of the malabaricones

The ability of the malabaricones to mediate cleavage of supercoiled (SC) DNA was assessed using agarose gel electrophoresis. The preliminary screening was carried out with mal A–D and the positive control, curcumin (each 25 μM). The gel electrophoresis pattern (Fig. 2) revealed that amongst the chosen compounds, only mal B, mal C and curcumin could induce the DNA cleavage in the presence of Cu(II) (100 μM). Mal C had the best DNA nicking ability, producing both open circular (OC) and linear (L) forms of DNA. Formation of the L form of DNA suggested extensive single strand breaks (ssb) in DNA. Mal B and curcumin were significantly less active and produced only OC form of DNA, while mal A and mal D were inactive (data not shown). No cleavage was observed under anoxic conditions. In separate experiments, we found that both Cu(II) and mal C are required to produce the DNA strand scission, while they were individually ineffective (Fig. 3a, lanes 2, 3 and 7). A concentration-dependent study on the effect of Cu(II) concentrations on the nuclease property of mal C (25 μM) revealed complete DNA nicking within 45 min with Cu(II) (100 μM) (lane 7), while the lower concentrations of Cu(II) were ineffective (lanes 4–6). Even after maintaining the mal C/Cu(II) ratio at 1:4, reduction of the mal C concentration (5 and 10 μM) led to less DNA nicking (data not shown). A time dependent study revealed formation of significant amount of the OC DNA by mal C (25 μM)–Cu(II) (100 μM) within 30 min. Thereafter, both OC and L forms of DNA were noticed with no trace of SC DNA. Beyond 60 min, smears of smaller heterogeneous DNA fragments were noticed on the gel (Fig. 3b). In view of these results, all subsequent studies were carried out using mal C (25 μM) and Cu(II) (100 μM) with an incubation period of 45 min.

To gain further insight into the mechanism of the DNA cleavage by mal C, the effect of various factors such as metal chelators and ROS scavengers in the process was studied. The gel electrophoresis pattern (Fig. 3c) revealed that the metal chelator, EDTA (250 μM) and the Cu(I)-specific chelator, neocuproine (250 μM) prevented the cleavage (lanes 5 and 6, respectively). The hydroxyl radicals scavenger, mannitol (100 mM) and superoxide dismutase (SOD, 250 U/ml) could not prevent the DNA cleavage (lanes 7 and 9). These results excluded the participation of the freely diffusible hydroxyl and superoxide radicals in the reaction. Additional experiments with higher concentration of mannitol (500 mM) and SOD (500 U/ml) as well as another hydroxyl radicals scavenger, DMSO (100 mM) also confirmed these. However, the participation of

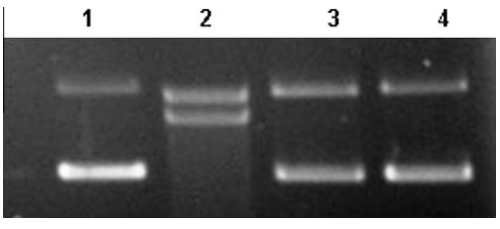


Figure 2. Ability of mal B, mal C and curcumin to induce Cu(II)-mediated damage of plasmid DNA. pBR322 plasmid DNA was incubated with the test compounds (each 25 μM) and Cu(II) (100 μM) in 10 mM potassium phosphate buffer (pH 7.4) containing NaCl (10 mM) for 90 min at 37 °C. Lane 1: pBR 322 DNA; lane 2: pBR 322 DNA + Cu(II) + mal C; lane 3: pBR 322 DNA + Cu(II) + mal B; lane 4: pBR 322 DNA + Cu(II) + curcumin. Other details are provided in Section 4.

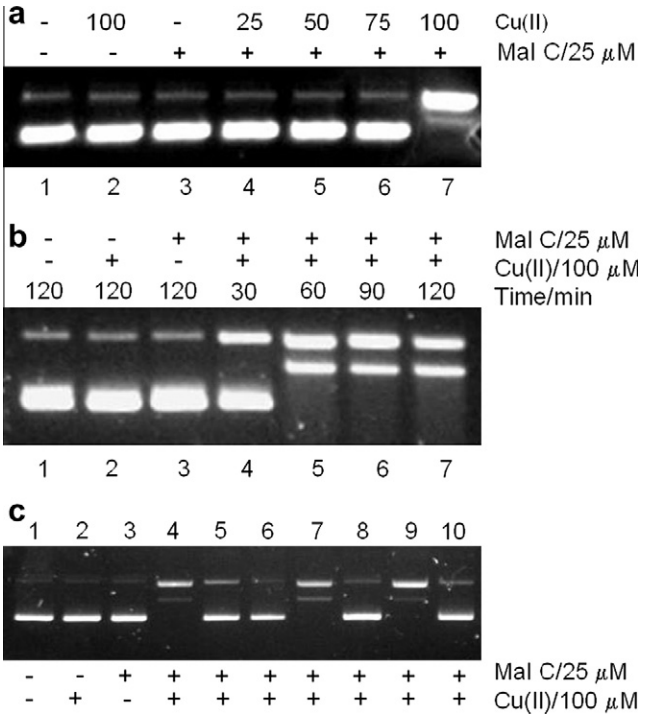
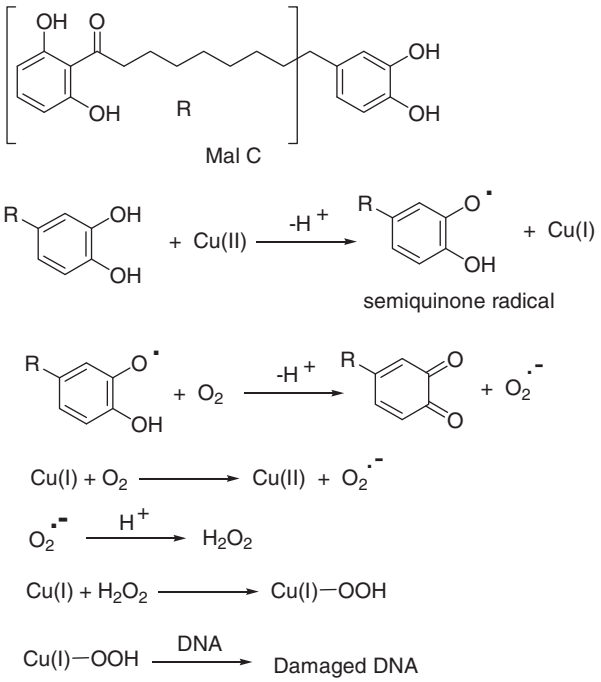


Figure 3. Effects of various parameters on the nuclease property of mal C. (a): Dependency of Cu(II) ion dose. pBR322 plasmid DNA was incubated with mal C (25 μM) and different concentrations of Cu(II) in 10 mM potassium phosphate buffer (pH 7.4) containing NaCl (10 mM) for 90 min at 37 °C. Other details are provided in Section 4. (b): Dependency of incubation time. pBR322 plasmid DNA was incubated with mal C (25 μM) and Cu(II) (100 μM) in 10 mM potassium phosphate buffer (pH 7.4) containing NaCl (10 mM) for different time periods, except the control samples. Other details are provided in Section 4. (c): Effect of ROS scavengers and metal ion chelators. Plasmid DNA was incubated with mal C (25 μM) and Cu(II) (100 μM) in 10 mM potassium phosphate buffer (pH 7.4) containing NaCl (10 mM) for 90 min at 37 °C in the presence of various reagents. Lane 1: pBR 322 DNA; lane 2: pBR 322 DNA + Cu(II); lane 3: pBR 322 DNA + mal C; lane 4: pBR 322 DNA + mal C + Cu(II); lane 5: pBR 322 DNA + mal C + Cu(II) + EDTA (250 μM); lane 6: pBR 322 DNA + mal C + Cu(II) + neocuproine (250 μM); lane 7: pBR 322 DNA + mal C + Cu(II) + mannitol (100 mM); lane 8: pBR 322 DNA + mal C + Cu(II) + NaN_3 (100 mM); lane 9: pBR 322 DNA + mal C + Cu(II) + SOD (250 U/ml); lane 10: pBR 322 DNA + mal C + Cu(II) + catalase (500 U/ml). Other details are provided in Section 4.

any site-specific and/or some copper-bound superoxide that can dismutate to hydrogen peroxide can not be excluded. On the other hand, the singlet oxygen ($^1O_2$) quencher, sodium azide[7a] (100 mM) and catalase (500 U/ml), the enzyme responsible for the disproportionation of H_2O_2, completely inhibited the DNA damage (lanes 8 and 10). In quantitative terms, the protections offered by some of the factors are as follows: EDTA (76.2%), neocuproine (98.5%), NaN_3 (93.1%), catalase (82.0%). These suggested the involvement of $^1O_2$ and H_2O_2 in the oxidative DNA damage by mal C–Cu(II) combination.

2.2. Mechanism of metal dependent DNA nicking by mal C

Based on these results, it is clear that the presence of Cu(II) and oxygen is the primary requirement of the DNA cleaving property of mal C. The cleavage is oxidative and does not require any external reducing agent. The data also indicated that Cu(I) is an obligatory intermediate of the process which appears to proceed via a Cu(I)-peroxo complex as is reported with many phenolics[7] and some polypyrroles.[8] Mechanistically the entire process can be depicted as shown in Scheme 1. In short, Cu(II) gets reduced by mal C to Cu(I), which on reaction with oxygen produces the superoxide ($O_2^{-\cdot}$) radicals and regenerates Cu(II). During this process, the catechol moiety of mal C gets converted into the semiquinone



Scheme 1. Pathway for the formation of Cu(I)-peroxo complex by mal C–Cu(II) combination.

radical. Its further oxidation to the quinone by molecular oxygen can also generate the $O_2^{\cdot-}$ radical anions. Eventually, disproportionation of the $O_2^{\cdot-}$ radicals furnishes H_2O_2, which on subsequent reaction with Cu(I) produces the Cu(I)-peroxo complex as the DNA cleaving agent. The formation of singlet oxygen in the process can be rationalized on the basis of the well-known copper mediated Fenton reaction as follows:

$$O_2^{\cdot-} + H_2O_2 \rightarrow {}^{\cdot}OH + {}^{-}OH + {}^1O_2$$

A few points emerged from the above results. The inactivity of mal A and mal D established that the resorcinol moiety in the 'A' ring of the malabaricones did not contribute to the nuclease property. However, presence of a phenol, and even better, a catechol moiety as the 'B' ring of the malabaricones is essential for the nuclease property. The phenolic group of the resorcinol moiety would be unavailable due to hydrogen bonding with the ketone moiety, and hence may not participate in the redox cycling of the Cu(II) ions. Also, Cu(II) can form a redox-inactive and stable chelate with the adjacent resorcinol and ketone moieties of the malabaricones, accounting for the inactivity of mal A and mal D. The requirement of Cu(II)/mal C in 4:1 ratio also suggested the second possibility. Between mal B and mal C, the latter contains a catechol moiety that can reduce the Cu(II) ions more easily than a phenol group. This accounts for the better DNA nicking activity of mal C than mal B. Amongst the benzene diols, catechol-containing phytochemicals,[7a,b,d] and synthetic compounds,[7e] were found to possess higher metal-dependent nuclease activity. Our results are consistent with these reports.

In the light of the above mechanism, the nuclease activity of mal C is likely to be governed by its ability to (i) bind with Cu(II) ions and DNA, and (ii) generate H_2O_2 via the superoxide radicals. Hence, all these factors were studied so as to assess their roles individually or in combination in deciding the DNA cleavage property of mal C. The results are presented below.

2.2.1. Cu(II) chelation

Mal C has two absorption maxima at ~276 and 345 nm, both of which showed hypsochromic shifts on gradual addition of Cu(II) in increasing amounts (Fig. 4). In addition, the absorption peak at 276 nm was red shifted by 5 nm and a new absorption peak at ~468 nm also appeared. These data indicated possible formation of a Cu(II)-mal C complex, and confirmed the copper chelating ability of mal C.

2.2.2. DNA binding

This was studied using three different techniques. The absorption spectroscopic studies revealed efficient DNA binding by mal C, as incremental addition of double stranded CT-DNA (0–50 μM DNA base pair) to a fixed concentration of mal C led to gradual reduction in the intensity of the band at ~345 nm (Fig. 5a). In addition, formation of a new species, with the absorption maximum at 452 nm indicated an intercalative DNA binding by mal C. Similar results have been reported with the well-known DNA intercalator, coralyne.[9]

Further indication of interactive DNA binding by mal C was obtained by the quenching of the ethidium bromide (EB) fluorescence. Due to its strong intercalation between the adjacent DNA base pairs, EB emits intense fluorescence in the presence of DNA. The enhanced fluorescence can be quenched by the addition of a second intercalating molecule and the extent of fluorescence quenching can be used to quantify the binding of the second molecule with DNA.[10] The emission spectra of EB bound to DNA in the absence and presence of mal C are shown in Figure 5b. Significant quenching (24.4%, P <0.05) of the EB florescence due to the pretreatment of CT-DNA with mal C (0.2 mM) revealed its interactive DNA binding.

As a means of further clarifying the intercalative DNA binding by mal C, the viscosity of DNA solution in the presence of increasing concentrations of mal C was measured. A classical intercalative mode binding increases the overall DNA length due to an increase in the separation of base pairs at the intercalation sites. This results in a significant increase in the viscosity of DNA solution in the presence of an intercalator.[11a,b] In contrast, complexes that bind exclusively in the DNA grooves by partial and/or non-classical intercalation, cause less or no change in the viscosity of the DNA solution.[11c] Our results showed that gradual addition of mal C to the DNA solution increased the relative viscosity of the solution (Fig. 5c), confirming an intercalative mode of DNA binding by mal C. Evidently, a strong binding of mal C with Cu(II) and DNA, as revealed by our results would enhance its capacity to cleave DNA.

2.2.3. ROS generation

For this study, an equimolar mixture of mal C and Cu(II) was incubated for 20 min and the amounts of superoxide radicals and H_2O_2 were quantified, by the nitro blue tetrazolium (NBT)

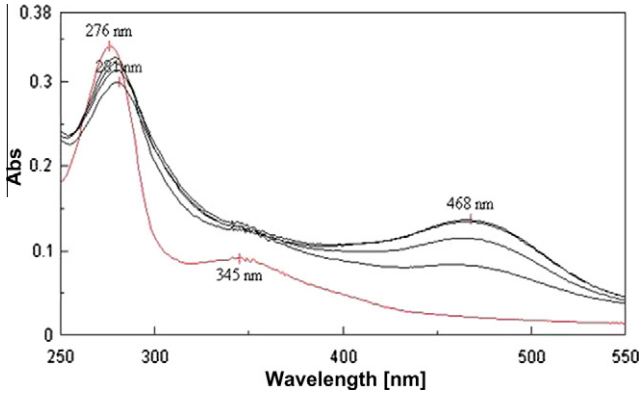


Figure 4. The absorption spectroscopic studies on Cu(II) chelation by mal C. The absorption spectra of mal C (25 μM) in HEPES buffer (10 mM, pH 7.2) were recorded in the absence and presence of $Cu(OAc)_2{\cdot}2H_2O$ (12.5, 25, 37.5 and 50 μM) at 25 °C. Red spectrum: mal C alone; black spectra: mal C + varying amounts of Cu(II).

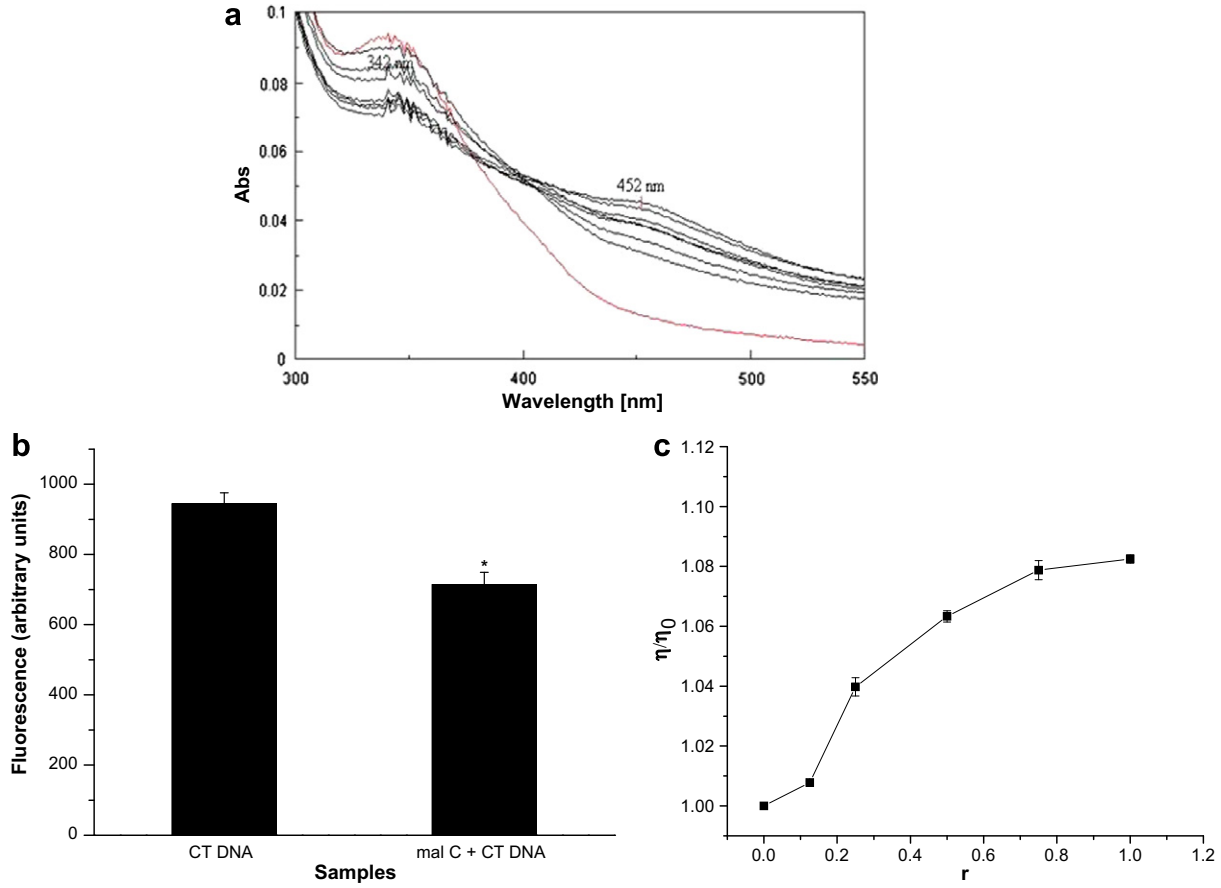


Figure 5. DNA binding by mal C. (a): By absorption spectroscopy. The absorption spectra of mal C (50 μM) in HEPES buffer (10 mM, pH 7.2) were recorded in the absence and presence of CT-DNA (3.125, 6.25, 12.5, 25, 37.5 and 50 μM) at 25 °C. Other details are provided in Section 4. Red spectrum: mal C alone; black spectra: mal C + different concentrations of CTDNA. (b): By EB fluorescence quenching. The fluorescence intensity of EB bound to CT-DNA in the presence and absence of mal C (0.2 mM) was measured at emission 600 nm after excitation at 530 nm. The reduction in fluorescent intensity is expressed as the percentage of that of control (CT-DNA + EB). The values are means ± S.E.M ($n = 4$). $^{*}P < 0.05$, compared to control. (c): By viscosity measurement. Viscometry of CT-DNA solution in the presence of different concentrations of mal C was measured using an Ubbelohde viscometer. Different R ([mal C]/[DNA base pairs]) values were obtained by addition of appropriate amounts of mal C to 0.2 mM CT-DNA solution in 10 mM Hepes buffer with 100 mM NaCl. The flow times of the samples were measured at least three times.

reduction[12] and horse radish peroxidase (HRPO)[13] assays, respectively. The NBT assay is based on measuring the absorbance at 560 nm of the chromogen formed due to its reduction by any reducing agent, such as $O_2^{-\cdot}$. Likewise, the H_2O_2 assay involves spectrophotometric measurement of the absorbance of the chromogen, produced by the HRPO-induced oxidation of phenol red with H_2O_2. True to the proposed hypothesis, the mal C–Cu(II) combination was found to increase the generation of $O_2^{-\cdot}$ radicals (~42, $P < 0.01$) and H_2O_2 (85%, $P < 0.001$) appreciably within 20 min, compared to the control value.

Taken together, these results validated the proposed mechanism of the Cu(II)-mediated nuclease activity of mal C. Apparently, in the presence of DNA, mal C and Cu(II) are located inside the DNA molecule, and generate the $O_2^{-\cdot}$ radicals close to DNA. The $O_2^{-\cdot}$ radicals get converted to H_2O_2 causing the DNA cleavage. Because the $O_2^{-\cdot}$ radicals are generated site specifically, the mal C-mediated DNA nicking would not be affected by SOD. However, the more stable ROS such as H_2O_2 and $^1O_2$, derived from the $O_2^{-\cdot}$ radicals can be effectively intervened by the suitable scavengers.

2.3. Inhibitory effects of mal A–D on proliferations of MCF-7 cells

It is well established that compounds that facilitate DNA nicking are promising candidates as cytotoxic and anti-tumour agents. The copper-dependent nuclease agents can cause lethal DNA damage due to the significant concentration of DNA-bound copper. A 3.5-fold increase in copper concentration has been reported in cancerous tissues compared to their non-cancerous counterparts.[14a] In a cellular system, the copper/H_2O_2 dependent damage is enhanced by packaging of DNA as a nucleosome.[14b] Breast cancer is the most common malignancy in women in both developed and developing countries of the world.[15] The epithelial breast cancer-derived MCF-7 cell line is a well-characterized estrogen receptor positive control cell line. Hence, in the present investigation, we used it as a model system and tested the anti-cancer property of the malabaricones, including mal C. Curcumin was chosen as the positive control in view of its structural similarity with that of the malabaricones, and due to its reported promise in cancer prevention and cure.[16] In addition, we have also used allyl pyrrocatechol (APC, chemical structure shown in Fig. 1), a simple catechol-containing compound, isolated from the *Piper betle* leaves, and assessed its cytotoxicity against the MCF-7 cell line.[17]

The cytotoxicity of the test compounds was assessed by the MTT assay.[18] Our results (Fig. 6A) revealed that except mal A, other test compounds possess impressive dose-dependent antiproliferative activity against the MCF-7 cells after a 72 h-incubation. Mal A showed marginal cytotoxicity (~12%) only at a high concentration (50 μM). The IC_{50} value for the antiproliferative activity of mal B–D and curcumin were 6.86 ± 2.26 μM, 5.26 ± 1.20 μM, 15.71 ± 2.63 μM and 24.46 ± 3.32 μM, respectively. Amongst the test compounds, the antiproliferative activity of mal B and mal C were

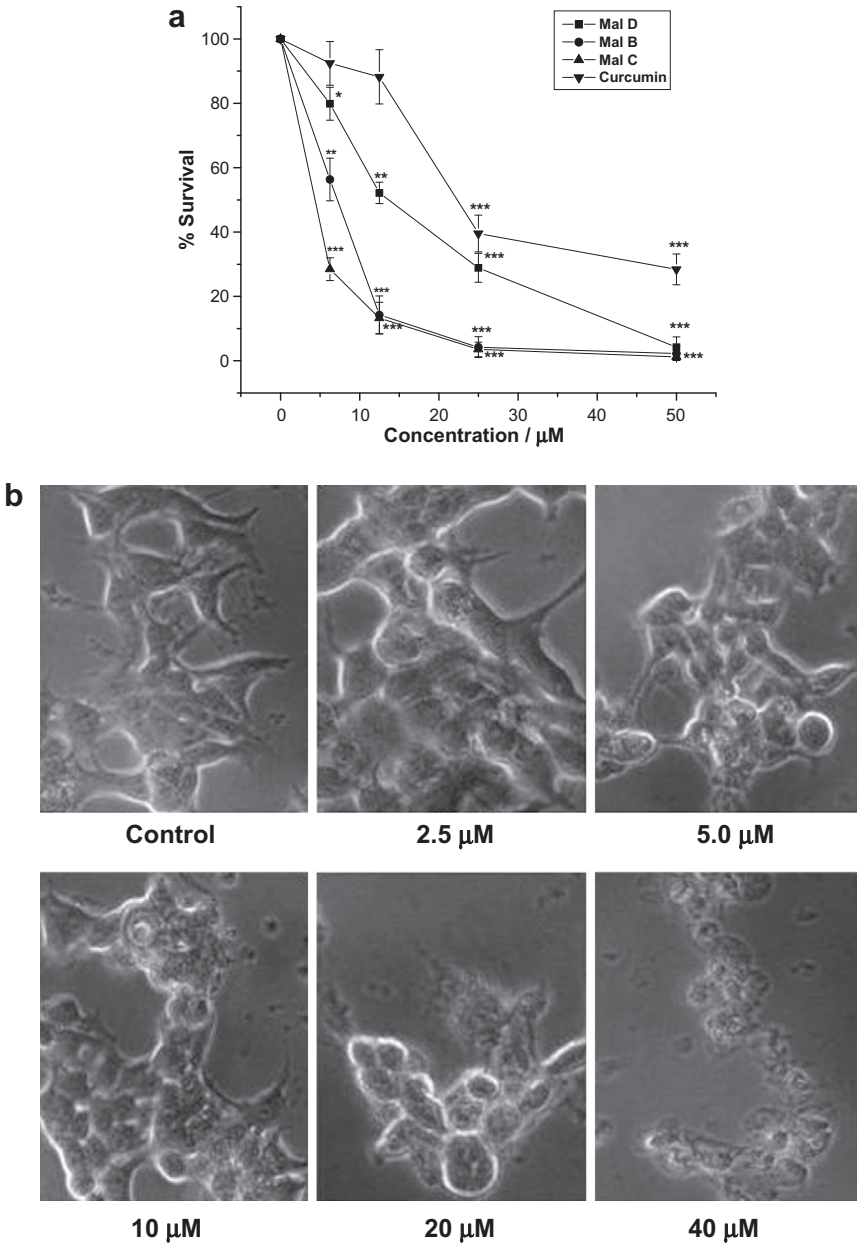


Figure 6. Dose-dependent growth inhibitory effect of the malabaricones against the MCF-7 cancer cells. (a): The cells grown in 96-well plates (10^4 cells/well) were treated with vehicle (0.1% DMSO) or increasing concentrations of mal A–D for 72 h. Cell viability was assessed by the MTT assay and the results are expressed in percentage considering that of the untreated control cells as 100. The experiments were repeated three times with similar results, all determinations were made in six replicates, and the values are means ± S.E.M. $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 compared to control cells. (b): Phase-contrast microscopy. The MCF-7 cells (1×10^5 cells/well) in 6-well plates were treated with vehicle (0.1% DMSO) or mal C (2.5, 5, 10, 20 and 40 μM) for 24 h, washed twice with PBS and visualized under a phase contrast microscope. The experiments were repeated three times with similar results, and a representative image is shown.

~3-fold that of mal D and 4–5-fold that of curcumin. The simple catechol, APC did not show any effect to the MCF-7 cell line even at a high concentration (100 μM).

The results clearly suggested that the presence of oxygenated substitutions in the aromatic B-ring of the malabaricones is crucial for their toxicity against the MCF-7 cells. Comparison of the results of mal B–D indicated that the presence of free phenolic hydroxyl groups in the B-ring augments the cytotoxicity. Interestingly, mal B and mal C, possessing a 4″-hydroxylated B-ring showed similar inhibitory potency against the MCF-7 cells. This is consistent with a previous finding where the 4″-hydroxyl group in the B-ring has been found to play a key role in the anti-cancer activity of resveratrol.[19] The excellent cytotoxicity of mal C was commensurate with its strong nuclease property, observed with isolated plasmid DNA. Interestingly, despite its poor in vitro prooxidant activity, mal B showed almost similar efficacy as that of mal C. Earlier, resveratrol (with an 4′-OH in its B-ring) was found[19b] to be converted to piceatannol (with two hydroxyls at 3′- and 4′-positions of B-ring) by a cytochrome P450 enzyme-mediated oxidation. Based on this report, it is tempting to propose a similar conversion of mal B to mal C for rationalizing the impressive anti-cancer property of mal B. Nevertheless, considering its best nuclease and anti-cancer property, mal C was used for all subsequent experiments.

The phase contrast microscopy, performed to examine the effect of mal C on morphological features on the MCF-7 cells, detected obvious morphological changes during concentration

dependent mal C-exposure.[20] A 24 h exposure to mal C-induced marked changes in the cell outline, with irregular disruptions in the optical diffraction halo (Fig. 6B). The number of shrinking cells or cells with blebbing membranes was notably increased in the mal C-treated group than in the control.

2.4. Apoptosis induction and ROS generation by mal C

Apoptosis plays an important role in the development and homeostasis of multi cellular organisms. Defective apoptosis is believed to be responsible in the pathogenesis of several clinical ailments, including cancer. Reduced apoptosis has been implicated in the development and progression of malignant tumors,[21a,b] and in the occurrence of chemoresistant phenotypes.[21c–e] Many studies have demonstrated that apoptosis induction plays the most vital role in the cancer treatment by chemo- and radiation therapy. Because apoptosis plays a critical role in cancer development and in the cellular response to anti-cancer agents, we investigated whether the mal C-induced cytotoxicity to MCF-7 cells followed an apoptotic pathway. For this, we assessed DNA laddering of the MCF-7 cells after treatment with mal C. As revealed from the gel electrophoresis photograph (Fig. 7), treatment of the cells with mal C produced DNA laddering dose-dependently. The laddering was prominent at a concentration of 10 μM of mal C.

Cancer cells are generally more active than normal cells in metabolic ROS generation and are constantly under oxidative stress.[22] Oxidative stress has been associated with many forms of programmed cell death and there is strong evidence that ROS can induce apoptosis.[23] Many agents that induce apoptosis are either oxidants, or stimulate oxidative metabolism. The anti-tumor activity of many important cytotoxic molecules such as sulforaphanes,[24a] doxorubicin,[24b] cisplatin,[24c] and emodin[24d] is reported to be mediated by ROS induction. Earlier, we have shown that the Cu-mediated nuclease activity of several hydroxystilbenes[25a] and anti-tumor property of a prodigiosin analogues[25b,c] are also mediated by ROS. Hence, we used the redox sensitive dye, 2′,7′-dichlorodihydrofluorescin diacetate (DCFDA) assay[26] to see if ROS is a potential factor in the mal C-induced cytotoxicity on the MCF-7 cells. Mal C was found to induce significant amount of ROS in the MCF-7 cells in a dose-dependent manner. The increase was from ~26% to 50% (P <0.01) with mal C 10 and 15 μM, respectively, compared to the control cells. Under similar conditions, the positive control, H_2O_2 (1 mM) increased the ROS level by 71% (P <0.001), compared to the control cells (Fig. 8). External supplementation of intracellular ROS scavengers like *N*-acetyl cysteine (NAC)[27a–c] is known to protect target cells against cytotoxic insults that are mediated through the participation of ROS. We envisaged if ROS mediates the apoptotic cell killing by mal C, the cells should resist its cytotoxicity in the presence of some added antioxidants. To confirm this, the MCF-7 cancer cells were pretreated with NAC (1 mM), prior to incubation with mal C (5 μM) for 72 h. Subsequently the proliferation of the treated cells was assessed by the MTT method. The NAC-treated cells showed significantly (P <0.05) more viability (75.32 ± 6.18%) than the control cells (45.32 ± 3.16%), after the mal C-treatment. These results indicated that mal C produced significant amount of intracellular ROS in the MCF-7 cancer cells, leading to its apoptotic death.

2.5. Induction of cellular DNA damage by mal C

The oxidative DNA damage by exogenous molecule is an important event in cellular system,[28] and if left unrepaired, may lead to cell death. Many anti-cancer agents induce DNA damage that triggers a p53 dependent apoptosis response. Hence, we used the comet assay to assess the mal C-induced DNA damage in the MCF-7 cells. Comet assay is one of the most sensitive and widely used techniques for detecting damage of cellular DNA.[29] In this assay, the comet tail length indicates the extent of DNA damage, because the cleaved DNA fragments move faster on the agarose gel. Figure 9 shows the comet gel of the untreated and mal C (5 μM)-treated cells. Our results showed that the DNA damage was negligible (comet tail: 1.25 ± 0.76 μm) in the untreated cells, which increased significantly (comet tail: 21.36 ± 5.28 μm, P <0.001) due to the mal C-treatment. The longer tail of the comet indicates that the DNA strand breaks were more frequent leading to several smaller fragments. The results clearly established that

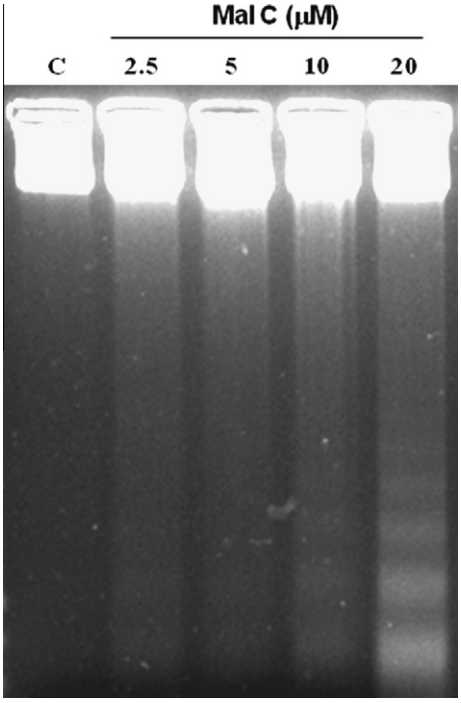


Figure 7. Dose-dependent apoptosis induction by mal C in the MCF-7 cells. The MCF-7 cells (1×10^5 per 60 mm dish) were treated with mal C (0–20 μM) for 24 h. The DNA from the cell lysate was analyzed on a 2% agarose gel and visualized after staining with ethidium bromide. Other details are provided in Section 4.

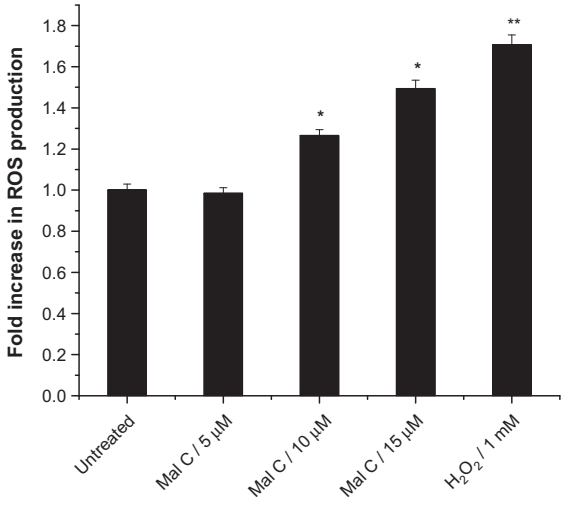


Figure 8. Dose-dependent ROS generation by mal C in the MCF-7 cells. Cells (1×10^5 cells/well) were treated with mal C (0–15 μM) or the positive control, H_2O_2 (1 mM) for 3 h. The amount of ROS generated in the cells was quantified by the DCFDA method. The experiments were repeated three times with similar results, all determinations were made in five replicates, and the values are means ± S.E.M. $^*P$ <0.01; $^*P$ <0.001 compared to the control untreated cells.

mal C was capable of DNA cleavage in the MCF-7 cells, which is in agreement with the cell viability results.

3. Conclusions

Overall, this is the first report of the Cu(II)-dependent DNA nicking ability as well as the anti-cancer property of the malabaricones, especially mal C. Our results showed that mal C possesses better potency than curcumin, against the MCF-7 cell line. The cytotoxic action of mal C against the MCF-7 cancer cells seems to be mediated by the mobilization of endogenous copper and the consequent prooxidant action. Cu(II) and Zn(II) ions are the major metal ions present in the nucleus, serum and tissues,[30] and in various malignancies the Cu(II) concentrations are greatly increased.[14] The Cu(II) ions from chromatin can be mobilized by metal chelating agents leading to increased ROS level. This, in turn, gives rise to internucleosomal DNA fragmentation. It has been shown that the curcumin-mediated apoptosis of HL60 cells is closely related to the increase in the concentrations ROS, generated through the reduction of transition metals in the cells.[31] The cellular DNA fragmentation by mal C that involves mobilization of intra-cellular and extra-cellular Cu(II), could be one of the mechanisms involved in its chemopreventive property. Mal C is abundant in rampatri which is extensively consumed in the eastern world without any side effect. Earlier we found that mal C is non-toxic to mice, even at an appreciably high dose (500 mg/kg).[5b] Considering this, and the encouraging present findings, mal C appears to be a potential anti-cancer agent for further evaluation. To this end, studies on its biodistribution and efficacy in a suitable animal model are being planned in our laboratory.

4. Experimental

Malabaricones A–D were isolated from the methanol extract of *M. malabarica* fruit rind and fully characterized from its spectral data as reported.[5a] Glutamine, fetal calf serum (FCS), calf thymus DNA (CT-DNA), Hepes buffer, superoxide dismutase (SOD), catalase, Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and Hepes buffer were procured from Sigma, St. Louis, MO. Other chemicals used were: ethylenediamine tetraacetic acid (EDTA) (Sarabhai Chemicals, Baroda, India), curcumin and $CuOAc_2{\cdot}2H_2O$ (Aldrich, Milwaukee, WI), nitro blue tetrazolium (NBT) and neocuproine (HiMedia, India), horse radish peroxidase (HRPO) and ethidium bromide (Sisco Research Lab., Mumbai, India), 35% H_2O_2 (Lancaster, Morecambe, UK) and pBR322 DNA (Bangalore Genei Ltd, India). CT-DNA was sonicated and phenol-extracted prior to use. The DNA concentration was determined using the molar extinction coefficient: $\varepsilon_{260} = 12824\ M^{-1}\ cm^{-1}$ in base pair (bp).[8a] Human MCF-7 (human breast cancer) cell line was procured from National Centre for Cell Sciences, Pune, India. Solutions of the test compounds were prepared in H_2O (up to 25 μM) or 2 mM cold aqueous NaOH (up to 100 μM).

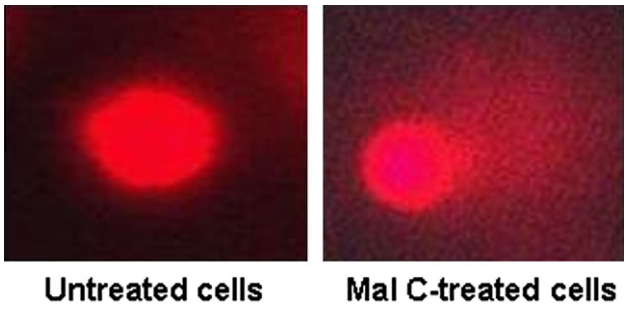


Figure 9. Single cell gel electrophoresis of MCF-7 cells, showing comet (40×) formation after treatment with mal C. Cells were treated with mal C (5 μM) for 2 h in the culture medium, subsequently washed two times with phosphate buffered saline, and processed for the comet assay. Other details are provided in Section 4. The experiments were performed three times and the number and overall tail spans of the comets in different areas were found to be consistently similar in all the experiments.

4.1. DNA nicking assay[32]

The reaction mixtures (final volume 13 μL) containing pBR322 plasmid DNA (200 ng) and $Cu(OAc)_2{\cdot}2H_2O$ (100 μM) in 10 mM potassium phosphate buffer (pH 7.4) containing NaCl (10 mM) were incubated at 37 °C for 45 min in the absence or presence of the test compounds. After adding the DNA gel loading dye (0.25% bromophenol blue, 50% glycerol and 500 μM EDTA), the samples were loaded in an agarose gel and subjected to electrophoresis at 72 V for 2 h. The DNA gels were stained with ethidium bromide (0.5 μg/mL) for 30 min, visualized under UV light using a Bio-Rad gel documentation system and the DNA bands quantified. Similar experiments were also carried out with different concentrations of mal C and $Cu(OAc)_2{\cdot}2H_2O$, individually or in combination. Experiments were also carried out in the presence of different factors (chelators, scavengers). For the time dependent experiments, incubation was carried out for 0–120 min.

4.2. Copper chelation study

Copper chelation study was carried out by recording the absorption spectra (190–600 nm) of a solution of mal C (0.5 mL, final concentration 25 μM) in HEPES (10 mM, pH 7.2) as such and 5 min after each addition of $Cu(OAc)_2{\cdot}2H_2O$ (final concentration up to 50 μM) solution in small aliquots. The copper chelation was evaluated from the change in absorbance and/or spectral shift.

4.3. DNA binding assay

4.3.1. Absorption method

The DNA binding capacity of mal C was determined by recording the absorption spectra (190–600 nm) of the compound (50 μM) in HEPES buffer (10 mM, pH 7.2) containing NaCl (10 mM), 5 min after addition of CT-DNA (final 12.5–50 μM) in small aliquots (0.164 mM) at 25 °C. The DNA binding by mal C was assessed from the change and/or spectral shift of the absorbance.

4.3.2. Quenching of EB fluorescence[10]

The experiments were done with a 1 mg/ml stock solution of CT-DNA in 10 mM Hepes buffer containing 100 mM NaCl. The concentration of DNA was determined from its absorbance at 260 nm using the molar absorption coefficient ($6600\ M^{-1}\ cm^{-1}$). An aliquot of EB (0.2 mM) was added to CT-DNA (0.2 mM) in the above buffer and the fluorescence at 600 nm was measured after excitation at 530 nm. In a similar experiment, the DNA was incubated with mal C (0.2 mM) for 30 min prior to the addition of EB. The fluorescence of DNA-bound EB in the absence and presence of mal C was measured.

4.3.3. Viscosity measurement[11]

The viscosity of CT-DNA solutions was measured at 30 ± 1 °C using an Ubbelohde viscometer. Typically, 1.0 mL of 10 mM Hepes buffer with 100 mM NaCl was transferred to the viscometer to obtain reading of the flow time. For determination of the solution viscosity, 1.0 mL of buffered solution of 0.2 mM CT-DNA was taken and the flow time reading was obtained. Different concentrations of mal C were added to the CT-DNA (0.2 mM) solution in the same buffer to give the appropriate r (r = [mal C]/[DNA]) values. The flow times of the samples were measured after achieving a thermal

equilibrium (30 min). Each measured point was the average of three readings. The data obtained are presented as (η/η_o) versus r, where η and η_o are the reduced specific viscosities of CT-DNA in the presence and absence of mal C.

4.4. Assay of superoxide produced by mal C and Cu(II)[12]

The reaction mixture (total volume 0.8 mL) contained mal C (100 μM), NBT solution (0.2 mL, 2 mM), NaCl (10 mM) and KH_2PO_4–KOH (50 mM, pH 7.4) buffer. The reaction was initiated by the addition of $CuOAc_2$ (100 μM) and incubating the mixture at 37 °C for 0 or 20 min. The amount of reduced NBT was read from the absorbance of the solution at 550 nm against the appropriate blank.

4.5. Assay of H_2O_2 produced by mal C and Cu(II)[13]

The reaction mixture (0.8 mL) containing KH_2PO_4–KOH (10 mM, pH 7.4) buffer, NaCl (10 mM), mal C (100 μM) and Cu(II) (100 μM) was incubated at 37 °C for 0 or 20 min. A solution (0.2 mL) containing NaCl (140 mM), dextrose (5.5 mM), phenol red (0.28 mM) and HRPO (8.5 U/mL) was added. After incubating for 5 min at room temperature, aqueous NaOH (10 μL, 1 N) was added to the mixture and the absorbance of the chromogen at 610 nm was read against an appropriate blank. The concentration of H_2O_2 stock solution was calculated from its absorbance at 230 nm, using an extinction coefficient of 81 M^{-1} cm^{-1}.

4.6. Cell culture

The cells were routinely seeded at a density of 0.1–3 × 10^6 cells/mL and grown in DMEM medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. Cells were passaged every 3–4 days to maintain 80–90% confluency. The cell viability was determined by the trypan blue dye exclusion assay. Subcultures were obtained by trypsinization with 0.25% trypsin in phosphate buffered saline (PBS).

4.7. Assay of cytotoxicity of mal A–D against MCF-7 cancer cells

The MCF-7 cells (1 × 10^4 cells per well) were grown overnight in 96-well microtiter plates in a regular serum containing medium. The medium was removed by aspiration, the cells were washed with PBS and 200 μL of medium (without FCS) containing different concentrations of the test compounds were added. After 3 h, FCS was added to the plates and incubation continued at 37 °C in 5% CO_2 for 72 h. The medium was removed by aspiration, the cells were washed twice with PBS, and the number of viable cells was determined using the MTT assay.[18]

4.8. Study of MCF-7 cell morphology

The MCF-7 cells (1 × 10^5 cells per well) in 6-well microtiter plates, grown on cover slips were incubated with different concentration of mal C (2.5–40 μM) for 24 h. The cells were washed two times with PBS and visualized under a phase contrast microscope. Representative fields of cells were photographed using a Zeiss Axioskop 2 mot plus microscope (40 × objective, 0.65 Ph2), fitted with a Axiocam MRc camera.

4.9. Apoptosis assay by DNA laddering

The mal C-induced apoptosis in the MCF-7 cells was monitored from the extent of chromatin fragmentation.[33] The MCF-7 cells (1 × 10^5 per 60 mm dish) were seeded and treated with mal C (0–20 μM) for 24 h. The harvested cells were washed with PBS and lysed at room temperature for 15 min with a lysis buffer containing 0.5% Triton X-100, 20 mM Tris, and 15 mM EDTA. The lysate was treated with RNase (0.1 mg/ml) and proteinase K (1 mg/ml) for 1 h and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated by incubating the upper aqueous phase with 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume of isopropyl alcohol overnight at −20 °C. The pellet obtained on centrifugation was washed with 70% ethanol, air dried and dissolved in Tris–EDTA buffer (50 μl). The extracted DNA was analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

4.10. Measurement of cellular ROS level[26]

The ROS levels were estimated from the ROS-mediated fluorescence enhancement of the cell permeable oxidation sensitive probe, dichlorofluorescein diacetate (DCFH-DA). The cells (1 × 10^5 per well), incubated with mal C (5–15 μM) or H_2O_2 (1 mM) for 3 h were scraped, centrifuged at 800*g*, and washed two times with PBS. After incubating with DCFH-DA (final concentration 5 μM) in PBS for 30 min at 37 °C, the cells were washed two times with cold PBS and lysed in PBS containing 1% Tween 20. The ROS level in the mixture, expressed as arbitrary units was determined from the DCF fluorescence (excitation at 480 nm and emission at 530 nm) of the untreated and differently treated cells.

4.11. Comet assay

Alkaline single-cell gel electrophoresis was conducted according to the reported method,[29a] with minor modifications. The MCF-7 cells (0.5 × 10^5), treated with mal C (5 μM) in DMEM medium and incubated for 2 h were washed with PBS and suspended in the same medium (10 μL). Frosted slides, coated with 80 μL of 0.5% high melting agarose was layered with 100 μL of low melting point (LMP) agarose (0.8% in PBS) containing 0.5 × 10^5 cells at 40 °C. Cover slips were placed immediately and the slides were placed on ice. After solidification, the cover slips were removed and each slide was coated with another layer of 100 μL LMP (0.8%) and kept at 4 °C as above. After removing the cover slip, the slides were placed in the lysing solution containing 2.5 M NaCl, 100 mM Na_2EDTA and 10 mM Tris–HCl, pH 10.0 with freshly added 1% Triton X-100 and 1% sodium sarcosinate for 1 h at 4 °C. The slides were removed from the lysis solution, washed three times with alkaline electrophoresis buffer and placed on a horizontal electrophoresis tank, filled with freshly prepared alkaline buffer (300 mM NaOH, 1 mM Na_2–EDTA, 0.2% DMSO, pH 13.0). The slides were equilibrated in the same buffer for 20 min and subjected to electrophoresis for 25 min at 1 V/cm. The slides were washed gently with 0.4 M Tris–HCl buffer, pH 7.4 to remove alkali, stained by layering on the top with 20 μL of propidium iodide (20 μg/mL), and visualized with a Carl Zeiss Axioskop microscope with bright field, phase contrast and epi-fluorescence facility (HBO 50 high-pressure mercury lamp), 0.5× camera adaptor lens, high-performance monochrome CCD camera (modified monochrome Cohu 49–12).

4.12. Statistical analysis

The values are expressed as the mean ± S.E.M. The data were analyzed by a paired Student's t test for the paired data, or one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparisons post hoc test.

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