Comparative effects of curcumin and its synthetic analogue on tissue lipid peroxidation and antioxidant status during nicotine-induced toxicity

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ABSTRACT

Introduction: Tobacco consumption is one of the leading preventable causes of death and disease worldwide. Nicotine, a major toxic component of tobacco, has been identified as an important risk factor for lung-related diseases. Increasing evidence demonstrates that oxidative stress plays a crucial aetiological role in the development of lung-related diseases. The present study aims at evaluating the protective role of curcumin and a synthetic analogue of curcumin (BDMC-A) on nicotine-induced oxidative stress.

Methods: Male albino rats of Wistar strain were used for the experimental study. Lung toxicity was induced by subcutaneous injection of nicotine at a dose of 2.5 mg/kg body weight (five days a week, for 22 weeks) and curcuminoids were given simultaneously by intragastric intubation for 22 weeks. Measurement of lipid peroxidation indices, thiobarbituric acid reactive substances and hydroperoxides, nitric oxide and antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, vitamin E and vitamin C, were used as biomarkers for testing the antioxidant potential of the drugs.

Results: Oxidative stress, as evidenced by lipid peroxidation indices, was significantly increased in nicotine-treated groups. Administration of curcumin and BDMC-A abrogated this effect. The antioxidant status which was decreased in nicotine was effectively modulated by both curcumin and BDMC-A treatment. However, the reduction in oxidative stress was more pronounced in BDMC-A treatment groups compared to those treated with curcumin.

Conclusion: The present study suggests that BDMC-A exerts its protective effect by modulating the extent of lipid peroxidation and augmenting the antioxidant defence system.

Keywords: antioxidants, curcumin, curcumin analogue, lipid peroxidation, lung toxicity, nicotine

INTRODUCTION

Nicotine is a major pharmacologically-active substance in cigarette smoke and plays an important aetiological role in the development of cardiovascular disorders, pulmonary disease and lung cancer.(1) Nicotine has been known to result in oxidative stress by inducing the generation of reactive oxygen species (ROS) in the tissues. These ROS in turn are capable of initiating and promoting oxidative damage in the form of lipid peroxidation (LPO).(2) LPO is known to cause cellular injury by inactivation of membrane enzymes and receptors, depolymerisation of polysaccharides, as well as protein cross-linking and fragmentation.(3) The mechanisms of ROS generation by nicotine are not clear. However, it has been reported that nicotine is chemotactic for polymorphonuclear (PMN) leucocytes and enhances the responsiveness of PMN leucocytes to activated complement C5a, thus generating ROS.(4) Further, nicotine disrupts the mitochondrial respiratory chain leading to the increased generation of superoxide anions and hydrogen peroxide.(5) It has been shown that experimental lung toxicity induced by the administration of nicotine in Wistar rats resulted in...
enhanced LPO and production of DNA-adducts, thereby initiating carcinogenesis.\(^{(6)}\)

Medicinal plants and their active principles have attracted the focus of recent attention as potential antiperoxidative agents. Turmeric (Curcuma longa rhizomes), commonly used as a spice, is well documented for its medicinal properties in Indian and Chinese systems of medicine. It has been widely used for the treatment of several types of diseases.\(^{(7)}\) Epidemiological observations, though inconclusive, are suggestive that turmeric consumption may reduce the risk of some form of cancers and render other protective biological effects.\(^{(8)}\) These biological effects of turmeric have been attributed to its constituent curcumin (Fig. 1) that has been widely studied for its antioxidant, anti-inflammatory, anti-angiogenic, wound-healing and anti-cancer effects.\(^{(9)}\) Previous studies from our laboratory and other reports have revealed the protective role of curcumin.\(^{(10)}\) Although a lot of work has been reported in the literature on the potential use of curcumin as an antioxidant, the search for new derivatives is ongoing to develop compounds that have a better antioxidant activity. The efficacy of the product, a novel curcumin analogue (BDMC-A), has already been proven effective against colon cancer and diabetes mellitus.\(^{(11,12)}\) Since little or no work has been done to evaluate the therapeutic strategy of BDMC-A (Fig. 2), in the present study, we synthesised an ortho-hydroxy-substituted analogue of curcumin and compared its effects with curcumin over nicotine-induced oxidative damage using LPO, nitric oxide (NO) and antioxidants as biomarkers.

**METHODS**

Male Albino rats (Wistar strain), with body weight range of 120–140 g, were obtained from the Central Animal House, Department of Medicine, Annamalai University, Tamil Nadu, India. The animals were housed six to a polypropylene cage and provided with food and water ad libitum. The animals were maintained under standard conditions of temperature at 30°C and 50% humidity with an alternating 12 hour light/dark cycle. Animals were fed standard pellet diet (Agro Corporation, Bangalore, India) and maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the ethical committee of Annamalai University.

Nicotine, curcumin (65%–70% purity) and other fine chemicals were obtained from Sigma Chemical Company, St Louis, MO, USA. All other chemicals and reagents used were of analytical grade. BDMC-A was prepared by refluxing a mixture of acetyl acetone, salicylaldehyde and boric acid in dimethyl formamide with a few drops of catalyst, namely, 1:1 glacial acetic acid and diethanolamine, for five to six hours. The mixture was then added to 10% acetic acid and stirred for two to three hours. The crude yellow mass was filtered and purified using silica gel column chromatography, and the purity...
checked with thin layer chromatography and the spectral data verified according to earlier reports.\(^{13}\)

The animals were randomised into experimental and control groups, and divided into six groups of six animals each. Rats in Group 1 served as control. Rats in Group 2 received subcutaneous injection of nicotine 2.5 mg/kg body weight (in physiological saline), five days a week for 22 weeks.\(^{14}\) The dilution was done in such a way that 1 ml of physiological saline contained the required dose of nicotine. Rats in Group 3 were administered nicotine as in Group 2 as well as curcumin 80 mg/kg body weight (in 1 ml of 5% DMSO) daily using an intragastric tube for 22 weeks. Rats in Group 4 received curcumin alone as in Group 3. Rats in Group 5 received nicotine as in Group 2 and BDMC-A 80 mg/kg body weight (in 1 ml of 5% DMSO) daily using an intragastric tube for 22 weeks. Rats in Group 6 received BDMC-A alone as in Group 5. Simultaneously, animals in Group 1 received 1 ml of 5% DMSO in physiological saline. The dose of curcumin and BDMC-A used in our study was based on the previous reports.\(^{15}\)

The experiment was terminated at the end of 22 weeks and all animals were sacrificed by cervical dislocation after an overnight fast. Blood samples were collected in heparinised tubes and plasma was separated by centrifugation at 2,000 g for ten minutes. Tissues (lung, liver and kidney) were removed, cleared of blood and immediately transferred to ice-cold containers containing 0.9% sodium chloride for various estimations. A known amount of tissue was weighed and homogenised in appropriate buffer (10%) for the estimation of various biochemical parameters.

LPO, as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) and hydroperoxides (HP), were measured by the method of Niehaus and Samuelsson\(^{15}\) and Jiang et al.\(^{16}\) respectively. In brief, 0.1 ml of tissue homogenate (Tris-HCl buffer, pH 7.5) was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25M HCl and 15% trichloroacetic acid) and placed in a water bath for 15 minutes, cooled and centrifuged at room temperature for 10 minutes at 1,000 rpm. The absorbance of clear supernatant was measured against a reference blank at 535 nm. For HP, 0.1 ml of tissue homogenate was treated with 0.9 ml of Fox reagent (88 mg butylated hydroxytoluene, 7.6 mg xylene orange and 9.8 mg ammonium ion sulphate were added to 90 ml of methanol and 10 ml of 250 mM sulphuric acid) and incubated at 37°C for 30 minutes. The colour developed was read at 560 nm colorimetrically.

NO was estimated by using the Griess method.\(^{17}\) To 0.5 ml of tissue homogenate, 0.1 ml of sulphasalicylic acid was added and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. To 200 μl of the protein-free supernatant, 30 μl of 10% NaOH was added followed by 300 μl of Tris-HCl buffer and mixed well. To this, 530 μl of Griess reagent (0.3% N-1 [Naphthyl-ethylene-diamine-dihydrochloride] in distilled water + 3% Sulphanilamide in 1M HCl) was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540 nm.

Superoxide dismutase (SOD) was assayed utilising the technique of Kakkar et al.\(^{18}\) A single unit of enzyme was expressed as 50% inhibition of nitroblue tetrazolium (NBT) reduction/min/mg protein. Catalase (CAT) was assayed colorimetrically at 620 nm and expressed as mmoles of H₂O₂ consumed/min/mg protein as described by Sinha.\(^{19}\) The reaction mixture (1.5 ml) contained 1.0 ml of 0.01M phosphate buffer pH 7.0, 0.1 ml of tissue homogenate and 0.4 ml of 2M H₂O₂. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). Glutathione peroxidase (GPx) activity was measured by the method described by Rotruck et al.\(^{20}\) Briefly, the reaction mixture contained 0.2 ml of 0.4 M phosphate buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of tissue homogenate (homogenised in 0.4M phosphate buffer pH 7.0), 0.2 ml glutathione, 0.1 ml of 0.2 mM H₂O₂. The contents were incubated at 37°C for 10 minutes. The reaction was arrested by 0.4 ml of 10% TCA, and centrifuged. Supernatant was assayed for glutathione content by using Ellman's reagent.

Reduced glutathione (GSH) was determined by the method of Ellman.\(^{21}\) 10% TCA was added to the homogenate and the mixture was centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412 nm. Vitamin C was estimated by the Roe and Kuether method.\(^{22}\) To 0.5 ml of tissue homogenate, 1.5 ml 6% TCA was added and centrifuged; and to the supernatant, acid-washed norit was added and filtered. To the filtrate, 0.5 ml of dinitrophenyl hydrazine was added and incubated at 37°C for three hours, and then 85% H₂SO₄ was added and further incubated for 30 minutes. The colour developed was read at 540 nm.

Vitamin E was estimated by the Baker et al method.\(^{23}\) Lipid extract was prepared by the method of Folch et al.\(^{24}\) To 0.5 ml of lipid extract, 1.5 ml ethanol and 2.0 ml of petroleum ether was added and centrifuged. The supernatant was evaporated to dryness at 80°C and to that, 0.2 ml of 2-2’ dipyril solution (0.2%) and ferric chloride (0.5%) were added, kept in the dark for five minutes, and then 4 ml of butanol was added. The colour developed was read at 520 nm. The total protein was estimated by total protein and albumin kit (No-72111, Qualigens Fine Chemicals, Worli, Mumbai, India).
For each tissue, values not sharing a common superscript letter differ significantly at \( p < 0.05 \) (Duncan’s multiple range test).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>TBARS (nM/100g)</th>
<th>Hydroperoxides (nM/100g)</th>
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<tr>
<td></td>
<td></td>
<td>Lung</td>
<td>Liver</td>
</tr>
<tr>
<td>1.</td>
<td>Control</td>
<td>0.65 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.70 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.</td>
<td>Nicotine</td>
<td>1.26 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.19 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.</td>
<td>Nicotine + curcumin</td>
<td>0.79 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.84 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.</td>
<td>Curcumin</td>
<td>0.58 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.71 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.</td>
<td>Nicotine + BDMC-A</td>
<td>0.72 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.79 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6.</td>
<td>BDMC-A</td>
<td>0.52 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.65 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
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<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>GSH (mg/100 g)</th>
<th>GPx (μm of GSH utilised/mg protein)</th>
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<tr>
<td></td>
<td></td>
<td>Lung</td>
<td>Liver</td>
</tr>
<tr>
<td>1.</td>
<td>Control</td>
<td>118.07 ± 7.96&lt;sup&gt;c&lt;/sup&gt;</td>
<td>127.69 ± 9.72&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.</td>
<td>Nicotine</td>
<td>73.89 ± 4.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>82.19 ± 6.42&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.</td>
<td>Nicotine + curcumin</td>
<td>93.82 ± 5.45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>106.48 ± 4.36&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.</td>
<td>Curcumin</td>
<td>124.77 ± 7.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>132.72 ± 8.34&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.</td>
<td>Nicotine + BDMC-A</td>
<td>103.68 ± 6.72&lt;sup&gt;c&lt;/sup&gt;</td>
<td>115.87 ± 6.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6.</td>
<td>BDMC-A</td>
<td>137.87 ± 9.98&lt;sup&gt;c&lt;/sup&gt;</td>
<td>140.98 ± 10.06&lt;sup&gt;c&lt;/sup&gt;</td>
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RESULTS

The extent of LPO as evidenced by the formation of TBARS, HP and the level of NO in lung, liver and kidney of control and experimental animals in each group are shown in Tables I and II. The levels of TBARS, HP and NO were significantly elevated in animals treated with nicotine when compared to controls. Administration of curcumin and BDMC-A to nicotine-treated animals (Groups 3 and 5) significantly decreased the level of TBARS, HP and NO when compared with nicotine-treated animals (Group 2). The enhancing effect was more significant in nicotine + BDMC-A-treated animals than nicotine + curcumin-treated animals.

The activities of SOD and CAT in the lung, liver and kidney of animals in each group are shown in Table III. The activities of SOD and CAT in the lung, liver and kidney were significantly decreased in nicotine-treated rats as compared to controls. These antioxidant activities were significantly raised in rats treated with nicotine + curcumin (Group 3), curcumin alone (Group 4), nicotine + BDMC-A (Group 5) and BDMC-A alone (Group 6) when compared to the nicotine group. However, nicotine + BDMC-A treatment was more effective than nicotine + curcumin treatment. The levels of GSH and the activity of GPx in the lung, liver and kidney of control and experimental animals are shown in Table IV. The level of GSH and the activity of GPx in the lung, liver and kidney were significantly depleted in nicotine-treated rats (Group 2) which were elevated on treatment with both curcumin and BDMC-A (Groups 3 and 5). The increase was more significant in BDMC-A-treated groups.

Table V shows the levels of vitamin E and vitamin C in the lung, liver and kidney of animals in each group. The levels of vitamin E and vitamin C in the lung, liver and kidney were significantly lowered in nicotine-treated animals (Group 2) compared with control animals, whereas the levels were significantly increased in both nicotine + curcumin (Group 3) and nicotine + BDMC-A (Group 5)-treated animals compared with nicotine-treated animals. However, the protective effect was more significant in nicotine + BDMC-A-treated animals when compared to nicotine + curcumin-treated animals.

DISCUSSION

It is well established that human patients and experimental animals exposed to nicotine display biochemical signs of oxidative damage. Evidences have indicated that free radicals or ROS, such as β-hydroxy ethyl radical, superoxide radical, hydroxy radical, peroxyl radical and hydrogen peroxide, are implicated in nicotine-induced oxidative tissue injury. Nicotine, a potential carcinogen, used in the present study has been reported to be oxidised into its metabolite cotinine, formaldehyde and 4- (methylnitrosamino)-1-(3-pyridyl)-1-butanone mainly in the liver and to a significant extent in the lung and kidney, and plays a key role in the pathogenesis of tissues. Mostly, nicotine undergoes 5'-hydroxylation induced by CYP2A6 to form cotinine and related metabolites, including formaldehyde giving rise to ROS. Thus, excessive generation of ROS as a consequence of induction of cytochrome CYP2A6 by nicotine plays a major role in the development of LPO and formation of lipid peroxidative end products. These findings support the elevation in TBARS and HP in the nicotine-treated group in our study.

NO plays an important role in a diverse range of physiological processes. NO reacts with the superoxide anion to generate peroxynitrite, which is a selective oxidant, and nitrating agent that interacts with numerous biological molecules, thereby damaging them. In our study, we have observed an increased level of NO in the lung, liver and kidney of nicotine-treated rats. Various factors in nicotine metabolism, such as the induction of CYP2A6 by nicotine play a role in the development of LPO and formation of lipid peroxidative end products. These findings support the elevation in TBARS and HP in the nicotine-treated group in our study.

Data on biochemical investigation were analysed using analysis of variance, and the group means were compared by Duncan’s Multiple Range Test. The results were considered statistically significant if the p-value was less than 0.05.

Table V. Levels of vitamin E and vitamin C in the lung, liver and kidney (mean ± SD; n=6).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Vitamin E (mg/100 g)</th>
<th>Vitamin C (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lung</td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>1.</td>
<td>Control</td>
<td>0.83 ± 0.05</td>
<td>0.75 ± 0.04</td>
</tr>
<tr>
<td>2.</td>
<td>Nicotine</td>
<td>0.52 ± 0.03</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>3.</td>
<td>Nicotine + curcumin</td>
<td>0.71 ± 0.04</td>
<td>0.69 ± 0.03</td>
</tr>
<tr>
<td>4.</td>
<td>Curcumin</td>
<td>0.88 ± 0.05</td>
<td>0.77 ± 0.04</td>
</tr>
<tr>
<td>5.</td>
<td>Nicotine + BDMC-A</td>
<td>0.75 ± 0.04</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td>6.</td>
<td>BDMC-A</td>
<td>0.92 ± 0.06</td>
<td>0.79 ± 0.04</td>
</tr>
</tbody>
</table>

For each tissue, values not sharing a common superscript letter differ significantly as p < 0.05 (Duncan’s multiple range test).
studies have shown that NO synthesis was high in tumour tissue and in plasma, which can be related to an alteration in the oxidant-antioxidant potential. Thus, a higher level of NO in the tissues of nicotine-treated rats could be due to the high production of free radicals by the toxic metabolites released during metabolism of nicotine.

There appears to be an inverse correlation between nicotine-induced LPO and antioxidant status. ROS generated in tissues are normally scavenged by enzymatic and non-enzymatic antioxidants. Antioxidant defence system protects the aerobic organism from the deleterious effects of reactive oxygen metabolites. Previous studies have suggested that superoxide anion and hydrogen peroxide are the main source of nicotine-induced free radical production depleting the cellular antioxidants. Glutathione is a crucial component of the antioxidant defence mechanism and it functions as a direct reactive free radical scavenger. The liver is the major organ with the highest content of GSH, which it supplies to the lungs and kidneys by a distinct GSH transport system. The decreased level of tissue GSH in nicotine-treated rats of the present study may be due to enhanced utilisation during detoxification of nicotine. GPx and CAT, which act as preventive antioxidants, and SOD, a chain-breaking antioxidant, play an important role in the protection against the deleterious effects of LPO. Depletion in the activities of SOD, CAT and GPx in the lung, liver and kidney of nicotine-treated rats may be due to decreased synthesis of enzymes or oxidative inactivation of the enzyme protein. Vitamin E, the major lipophilic antioxidant, and vitamin C, an essential water-soluble antioxidant, play a vital role in preventing the oxidative stress. In the present study, increased LPO associated with decreased vitamin C and vitamin E in the liver of nicotine-treated rats can therefore be related to insufficient antioxidant potential.

Administration of curcumin and BDMC-A significantly reduced the extent of LPO, NO and enhanced the antioxidant status in the lung, liver and kidney. The significant reduction in the levels of LPO products confirms that curcumin and BDMC-A could effectively protect against the free radicals. Curcumin, by scavenging or neutralising free radicals, interacting with the oxidative cascade, quenching oxygen, inhibiting oxidative enzymes like cytochrome P450, and chelating metal ions like Fe$^{2+}$, inhibits peroxidation of membrane lipids and maintain cell membrane integrity and function. Curcumin has been shown to inhibit LPO in rat liver microsome, erythrocyte, bronchoalveolar lavage fluid and brain homogenate. Curcumin has been reported to reduce nitrite formation during NO oxidation. The inhibitory effect of curcumin on the oxidation of NO to nitrite is due to its known sequestration of the reactive intermediate nitrogen dioxide systems. This suggests that administration of curcumin prevents the degradation of NO mediated by nicotine-induced oxygen free radicals.

The antioxidant effect of BDMC-A was shown to be more effective than curcumin. Among many classes of compounds, phenolics have been recognised as a powerful counter measure against LPO. Normally, phenolic compounds act by scavenging free radicals and quenching the lipid peroxidative side chain. It has been proposed that hydroxy and hydroperoxy radicals initiate hydrogen abstraction from a free phenolic substrate to form phenoxy radicals that can rearrange to quinone methide radical intermediates which is excreted via bile. BDMC-A, being a phenolic compound, might have inhibited LPO. Thus, curcumin and BDMC-A may significantly reduce the extent of LPO products and NO in the lung, liver and kidney. Moreover, administration of curcumin or BDMC-A to nicotine-treated rats enhanced the GSH level and increased the activities of SOD, CAT and GPx in the lung, liver and kidney. A previous study has shown that curcumin is a potent inducer of detoxifying enzymes and thereby prevents the toxicity induced by chemical carcinogens. BDMC-A is as efficient as curcumin in its inducing capacity of detoxifying enzymes. Sreejayan and Rao have also reported that BDMC-A is a potent inducer of detoxifying enzymes. Further, the presence of hydroxyl groups in the ortho position increases its antioxidant potential through intermolecular hydrogen bonding involving the –SH group of non-protein thiols and enzymes (Fig. 3). The effective antioxidant property of curcumin and BDMC-A decreases the utilisation of vitamin C and vitamin E in the liver and thus maintains their level.

![Fig. 3 Activation of sulfhydryl group by BDMC-A through intermolecular hydrogen bonding.](image-url)
The present study shows that novel synthetic curcuminoid may mediate its protective effect against nicotine-induced toxicity by modulating the extent of LPO and NO, and augmenting the antioxidant defence system. Further, our results suggest that synthetic curcuminoid may become a promising agent for the prevention and control of lung cancer development.

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40 Roe JM, Kuether CA. Detection of ascorbic acid in whole blood, and urine through the 2,4-DNPH derivative of dehydroascorbic acid. J Biol Chem 1943; 147:399-407.