Curcumin Scavenges Peroxynitrite in RAW 264.7 Macrophages through Phenolic Hydroxyl Groups

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Authors’ contributions
This work was carried out in collaboration among all authors. Authors MK and MM conceptualized the study. Authors MK and NMA designed the methods. Author MM validated the study. Authors MK, MM and NMA performed formal analysis. Authors MK and NMA involved in study investigation and obtained resources for the study. Authors MK, MM and NMA managed the data curation. Authors MK, MM, MSH and MIMI wrote, reviewed and edited the original draft. Authors MK, MM and NMA visualized the study and data presentation. Authors MK, MM and NMA managed the whole project. All authors read and approved the final manuscript.

ABSTRACT

Background: Overproduction of free radicals is implicated in cell death and tissue injury. Peroxynitrite (PN) is a highly oxidizing and short-lived free radical that is formed by the interaction of nitric oxide (NO) with superoxide. Curcumin is a natural compound obtained from Curcuma longa and has high antioxidant and anti-inflammatory activities.

Aim: We investigated the PN scavenging ability of curcumin to determine its potential as a therapeutic agent for chronic diseases caused by highly oxidative molecules.

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1. INTRODUCTION

The mammalian immune system protects the body from microbes or antigens continuously throughout the lifecycle. Microbes and other molecules are recognized by the immune system and are removed from the body by phagocytosis [1,2]. During the immune response, neutrophils and macrophages are activated and migrate to the site of damage where they produce highly oxidizing free radicals. These cells thus play critical roles in removing microbes and antigens by expressing high levels of cytotoxic molecules such as nitric oxide (NO) and reactive oxygen species (ROS) [3].

The source of NO during the immune response is inducible nitric oxide synthase (iNOS) that is proportionate with the level of immune response. iNOS is activated by bacterial lipopolysaccharides (LPS), the expression of which is correlated with the degree of inflammation [3-5]. The inflammatory modulators produced in response to LPS, NO, and other ROS are implicated in both acute and chronic inflammation [3,4,6,7], when there is extensive damage to host tissues. The damage is, in part, due to the reaction of NO with superoxide radicals, resulting in the formation of peroxynitrite (PN). Peroxynitrite is a key macrophage-derived cytotoxic agent released to the phagosomes of activated macrophages upon T. cruzi invasion and promotes protein tyrosine nitration in the parasite. PN is a short-lived oxidant that profoundly influences the inflammatory response at multiple cellular sites from the cell wall to the nucleus and is also a potent inducer of cell death [6,8]. The biological targets of PN include membrane, cytosolic, and nuclear receptors [3,9,10]. PN interacts with many inflammatory mediators including cytokines and iNOS during the immune response [9,11].

Several studies indicate that the majority of the adverse effects of excess NO generation on host immune response are caused by the formation of PN rather than NO itself. PN interacts with a wide range of biological molecules, including amino acids, leading to changes in protein structure and function [6,7,9]. PN also causes DNA damage and suppresses host defenses by oxidation of cellular thiol pools, lipid peroxidation, and nitration of the aromatic ring of free tyrosine and protein tyrosine residues. The most important reaction of PN is nitration of tyrosine residues. This is indicated by the presence of nitrotyrosine in tissues or cell cultures, which is often used as a marker of PN production.

Previous studies have implicated PN in diseases such as asthma, acute lung injury, idiopathic pulmonary fibrosis, inflammatory bowel disease, septic shock, arthritis, psoriasis, multiple sclerosis, systemic lupus erythematosus, Alzheimer’s disease, acute renal ischemia, and hyperlipidemia [3,5,12]. Pharmacological strategies aimed at attenuating the toxic effects of PN are inhibition and/or scavenging in order to limit tissue damage. Most natural products have antioxidant activities and some compounds derived from natural products have shown potent activity against oxidative stress. Natural products such as uric acid, apocynin, and caffeic acid phenethyl ester [4,9].

The rhizome of Curcuma longa is a curry spice with a long history of use in traditional herbal

Methodology: We examined the PN scavenging ability of curcumin either by directly incubating lipopolysaccharide (LPS)+interferon (IFN)-γ-stimulated RAW 264.7 murine macrophages with PN or indirectly through incubation with a PN donor (the artificial substrate SIN-1). Student t-test and one-way ANOVA were used to determine the statistical significance of differences between the experimental and control groups.

Results: The results demonstrate that curcumin inhibits PN and the synthesis of PN from SIN-1. Curcumin also significantly improved the viability of LPS+IFN-γ-treated RAW 264.7 macrophages and inhibited NO production. In macrophages, curcumin inhibited cellular PN synthesis, as evidenced by the absence of 3-nitrotyrosine, a marker of PN-oxidized proteins.

Conclusion: Curcumin attenuates the immune responses that lead to cellular damage and cell death through suppression or scavenging of cytotoxic molecules such as NO and PN. The role of the phenolic hydroxyl of curcumin is critical in PN scavenging.

Keywords: Peroxynitrite; nitric oxide; curcumin; lipopolysaccharide; free radical.
medicines. It contains one of the most active compounds, called curcumin, which has a yellow pigment. Curcumin shows anti-oxidant, free radical scavenging, and anti-inflammatory activities [13,14]. In this study, we investigated the PN scavenging ability of curcumin to determine its potential as a therapeutic agent for chronic diseases caused by highly oxidative molecules.

2. MATERIAL AND METHODS

2.1 Cell Culture and Reagents

The murine macrophage cell line RAW 264.7 was obtained from (ATCC No. TIB-71, American Type Culture collection, Rockville, MD, USA). Raw 264.7 were maintained in Phenol-Red-Free Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 100 U streptomycin/mL, 100 U penicillin/mL, and 10% (v/v) heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37°C and 5% CO2. Phenol red-free DMEM, FBS, and antibiotics were obtained from Nacalai Tesque (Kyoto, Japan). The inducible NO synthase (iNOS) inhibitor 1400W, LPS (Escherichia coli 0111 B4), and IFN-γ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Curcumin and dimethoxycurcumin (DMC) were purchased from Cayman Chemical Co. (Michigan, USA).

2.2 Dihydorhodamine-123 Oxidation Assay

This assay measured dihydrorhodamine-123 (DHR-123) oxidation under three different conditions. First, by incubation of curcumin with SIN-1 (3-morpholinosydnonimine) as the PN donor in the presence of DHR-123. Second, by direct incubation of curcumin with PN in the presence of DHR-123. Third, by incubation of live cells stimulated with LPS+IFN-γ with curcumin in the presence of DHR-123 as described in [8].

2.3 DHR-123 Oxidation Using SIN-1

SIN-1 spontaneously releases NO and superoxide under physiological conditions. At pH 7.4, SIN-1 is converted to SIN-1A via base-catalyzed ring opening. During ring opening, the oxygen undergoes univalent reduction to O2−, SIN-1A then releases NO and is converted to the stable metabolite SIN-1C, whereas the O2− radical reacts with NO to form peroxynitrite (ONOO−). The oxidation of DHR-123 by ONOO− results in the formation of fluorescent rhodamine 123, the amount of which can be measured by fluorometric analysis (GloMax®-Multi Microplate) at an excitation wavelength of 460–530 nm and an emission wavelength of 530–590 nm. In experiments examining the effects of curcumin (0.06-2 µg/mL) or DMC(2 µg/mL) or 100 µM of 1400W on peroxynitrite scavenging, 100 µM of SIN-1 was used and the reactions were carried out in PBS, with the incubation of the samples for 2 h at 37 °C.

2.4 DHR-123 Oxidation Using Peroxynitrite

The ability of peroxynitrite to oxidize DHR-123, thus converting it to rhodamine 123, was also measured directly as previously described. Briefly, 10 µM peroxynitrite was mixed in PBS containing 20 µM DHR-123, in the absence or presence of either curcumin (0.06-2 µg/mL) or DMC (2 µg/mL) or 100 µM of 1400W. After a 15-min incubation period at room temperature, the fluorescence of the rhodamine 123 reaction product was measured (GloMax®-Multi Microplate) at an excitation wavelength of 460–530 nm and an emission wavelength of 530–590 nm.

2.5 DHR-123 Oxidation by LPS+IFN-γ-Treated RAW 264.7 Cells

Cells were cultured, stimulated with LPS, and treated with curcumin or 1400W as described in subsection (LPS+IFN-γ stimulation of RAW 264.7 cells) in the presence of 10 µM DHR-123. After 24 h, 100 µL of the culture suspension was removed, and the amount of rhodamine 123 was determined fluorometrically.

2.6 Induction of RAW 264.7 Cells by LPS+IFN-γ

RAW 264.7 cultured in Phenol-Free DMEM with 10% FBS were seeded in 24-well plates at a density of 2 × 106 cells/well and incubated for 24 h at 37°C. The next day, cells were washed with PBS and re-suspended again in fresh phenol-free DMEM/10% FBS containing a range of concentrations of curcumin (0.0625–2 µg/mL) or 100 µM iNOS inhibitor 1400W. The control and untreated (LPS+IFN-γ only) were untreated in each experiment. All cells were incubated for a minimum of 1 h at 37°C in a 5% CO2 atmosphere. Subsequently, LPS (1 µg/mL) and IFN-γ (35 ng/mL) were added to the cultures [8]. The next day, all cells were analyzed to test cell viability or production of NO or PN, as described below.
2.7 Measurement of Cell Viability

The viability of RAW 264.7 cells was measured in all cultures, including those untreated or treated with a combination of LPS+IFN-γ and curcumin (0.0625–2 µg/mL) or the iNOS inhibitor 1400W (100 µM). The viability of all cells was detected based on cellular respiration by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Briefly, cells were cultured, treated with curcumin or 1400W, and then stimulated with LPS+IFN-γ as described above. One-hundred microliters of MTT (5 mg/mL) was then added to each well and incubation continued under the same cell culture conditions for 1 h. The medium with MTT solution was then removed and cells were solubilized in 200 µL DMSO by shaking for 5 min. Absorbance was measured at 550 nm using a microplate reader (GloMax®-Multi Microplate detection, Promega, Madison, WI, USA) [8]. All experiments were repeated five times in triplicate.

2.8 Measurement of Nitric Oxide

NO is rapidly converted to nitrite (NO2) and nitrate (NO3) due to its short half-life. The colorimetric Griess reaction was used to indirectly determine the NO concentration through measured NO2. Cells were cultured and treated with curcumin and 1400W then stimulated LPS+IFN-γ as described above, and then 100 µL of the culture supernatant was placed in a 96-well plate with an equivalent amount of the Griess reagent (50 µL of 1% sulfanilamide in 5% concentrated H3PO4 and 50 µL of 0.1% naphthylethylenediamine dihydrochloride in distilled water) then incubated 10 minutes at room temperature, protected from light. Absorbance was measured at 550 nm using a microplate reader. The reaction yields a pink derivative that can be spectrophotometrically quantified from a standard concentration curve prepared from an NO2 standard [8].

2.9 Measurement of 3-nitrotyrosine Levels in RAW 264.7 Cells

Lysates were prepared from RAW 264.7 cells collected after treatment as described above for the stimulation experiments and the level of 3-nitrotyrosine, a marker of PN, measured. The harvested cells were placed on ice and immediately lysed in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease-inhibitor cocktail. Lysates were then centrifuged at 16,000 ×g for 15 min at 4°C. The supernatants were transferred to pre-cooled tubes and stored at -80°C until analysis. Concentrations of 3-nitrotyrosine in the lysates were measured with a nitrotyrosine ELISA kit (Cell Biolabs, Inc, San Diego, CA) according to the manufacturer’s instructions. Nitrotyrosine levels were normalized to the amount of protein in milligrams. Protein concentrations were measured using the Bradford method.

2.10 Statistical Analysis

The data were analysed using SPSS software version 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). Student t-test and one-way ANOVA (with post-hoc test) were used to determine the statistical significance of differences between the experimental and control groups. Statistical significance was set at p ≤ 0.05.

3. RESULTS AND DISCUSSION

Effects of curcumin on cell viability and intracellular levels of PN in cells induced with LPS+IFN-γ.

The above results indicate that curcumin appears to directly scavenge PN without having an effect on PN donors. All of the doses selected and used for curcumin, and 1400W in this study did not reduce cell viability after a 24-h incubation compared with untreated (Fig. 1A). On the other hand, groups treated with curcumin and 1400W significantly protected cells from damage of immune responses induced by LPS+IFN-γ. Viability was ≥75.1% in cells induced with LPS+IFN-γ and treated with curcumin and 1400W compared with ≤60.3% in cells induced with LPS+IFN-γ and in cells untreated with curcumin or 1400W (Fig. 1B). In addition, curcumin and 1400W inhibited PN production in response to cell induction by LPS+IFN-γ. This was shown by reduced DHR-123 fluorescence in these cells compared with untreated cells (Fig. 1C). Furthermore, the LPS-induced immune response mediated accumulation of nitrate was significantly reduced by curcumin and 1400W when compared with untreated (Fig. 1D).
Additionally, curcumin and 1400W blocked intracellular PN formation, as confirmed by reduction in the level of 3-nitrotyrosine, an intracellular marker of PN (Fig. 2).

Fig. 1. (A) Cytotoxic effect of curcumin on the viability of cells. Cells (RAW 264.7) were treated with curcumin or 1400W or were untreated and then incubated for 24 h. The MTT assay was used to assess cell viability as described in Materials and Methods. (B) The protective effect of curcumin on cells stimulated with LPS+IFN-γ. Cell were incubated with curcumin or 1400W or left untreated 1 h before stimulation with LPS (1 μg/mL) and IFN-γ (35 ng/mL) and then incubated for 24 h. The MTT assay was used to assess cell viability as described in the Materials and Methods. (C) The inhibitory effect of curcumin on the levels of PN produced in the presence of DHR-123 after cells were induced with LPS+IFN-γ and then incubated for 24 h. Cells were treated as described in the Materials and Methods. DHR-123 oxidation of curcumin at various concentrations was significantly lower than that for the untreated, while 1400W completely inhibited DHR-123 oxidation (D) The inhibitory effect of curcumin on the level of nitrite production in cells incubated 24 h after induction with LPS+IFN-γ. Curcumin showed significant, dose-dependent inhibition of nitrite production, while 1400W treatment blocked nitrite production compared with cells treated with LPS+IFN-γ only. Each value represents the mean ± S.E.M. of five independent experiments. p ≤ 0.02 (*) p ≤ 0.003 (**) and p ≤ 0.0001 (***)
The inhibitory effect of curcumin on the intracellular levels of PN in cells stimulated with LPS+IFN-γ after treatment and incubation for 24 h. Treatment with the inhibitor of iNOS (1400W) and curcumin at the indicated concentrations showed significantly inhibited 3-nitrotyrosine production compared with the results for the untreated (treated with LPS+IFN-γ only). Each value indicates the mean ± S.E.M. p ≤ 0.0001 (***)

3.1 Inhibitory Effects of Curcumin on PN Release

The results clearly show that curcumin inhibited PN generation from the synthetic substrate SIN-1. However, the effects of DMC and 1400W were similar to those of the untreated (Fig. 3A). On other hand, the accumulation of nitrite produced from SIN-1 was not effected by curcumin, DMC (2 µg/ml), and 1400W (Fig. 3B). In addition, direct incubation of PN for short time (15 min) and curcumin reduced DHR-123 oxidation. Over this same time period of 15 min, incubation of PN with DMC, 1400W, and untreated did not reduce DHR-123 oxidation (Fig. 3C).

The mechanism of NO-mediated cell damage involves generation of highly oxidative molecules acting on multiple cellular targets, such as lipids, DNA and various proteins [15,10]. However, many previous studies have shown that exposure to high concentrations of PN often leads to rapid, necrotic-type cell death owing to acute and severe cellular energetic derangements [10,16,17]. However, the reduction of PN concentrations suspended apoptosis by activation of caspases 3, 2, 8, and 9 [18,19,20,21]. However, apoptosis via release of cytochrome c into the cytosol results in release of mitochondrial proapoptotic factors that subsequently trigger DNA fragmentation and the mitochondrial permeability transition pore, all of which are stimulated and oxidized by PN. The key role of PN in promoting mitochondrial dysfunction is clearly exemplified in high immune response models such as experimental endotoxemia and cecal ligation puncture, wherein PN production results in the inhibition of mitochondrial respiration in the diaphragm in a process associated with mitochondrial protein nitration [22]. Survival rate is improved in experimental cecal ligation puncture after using a PN blocker or a Nitric Oxide Systems (NOS) inhibitor, which is of importance because NOS is a source of PN in the body [23]. The pathway of MLK/p38/JNK is very important in the mechanism of apoptosis. PN is a strong inducer and activator of the MLK/p38/JNK pathway [24,
This study and previous studies showed inhibition of NO in cells and animals due to inhibition of iNOS, which is responsible for NO and PN production from cells during and after the immune response. However, inhibition of iNOS by curcumin or 1400W leads to reduction in NO and PN production, as well as reduction in damage and cytotoxicity. Moreover, scavengers of PN are known to be protective against tissue damage.

Earlier studies discovered some endogenous and exogenous scavengers of PN, such as mercaptalkylguanidines, ebselen, uric acid, dihydrolipoic acid, and N-acetylcysteine. Some chemicals, such as metalloporphyrins of iron and manganese, work as decomposition catalysts of PN and can attenuate the toxic effects of PN in vitro and in vivo [10,25-31]. During the immune response and in various pathophysiological conditions, all of the above scavengers can inhibit 3-nitrotyrosine, which has beneficial effects in animal models of inflammation and reperfusion injury [4,32-37]. In addition, phenolic compounds (such as phenic acid and flavonoids) and polyphenolic compounds (such as caffeic acid, ferulic acid, kaempferol, gallic acid, quercetin, and p-coumaric) have been shown to inhibit PN. While monohydroxylated phenolic compounds (such as p-coumaric and ferulic acid acid) act as PN scavengers via nitration, compounds with a catechol moiety (such as chlorogenic acid and caffeic acid) reduce PN production by electron donation [38,39].

Scavengers and neutralizers of PN are needed for treatment of the diseases described above in which PN is implicated. Many compounds mentioned above scavenge and react with PN directly or indirectly, depending on the structure of compounds [40]. In vitro study using DHR and its fluorescent oxidation product is one important method due to specific interaction with PN without interfering with NO and superoxide. It is an excellent indicator of PN scavenging. Our in vitro results showed the potential of curcumin to scavenge PN. At physiological pH, PN rapidly protonates to either generate peroxynitrous acid or reacts with CO2 to form nitrosoperoxycarbonate. Both PN products can nitrate proteins through phenol groups, particularly tyrosine. The presence of 3-nitrotyrosine in cell culture or serum is a specific biomarker of oxidation of proteins by PN. 3-nitrotyrosine has also been detected in organs after sepsis, reperfusion and ischemia [12,41,42]. Our results showed the ability of curcumin to inhibit protein oxidation by PN through reduction of levels of 3-nitrotyrosine in cell culture. During the immune response, cells undergo physiological changes and increase the activity that leads to increases in the levels of oxidative stress and free radicals, specifically (ROS). Increased activity of iNOS, heme oxygenase 1 (HO-1), cytokines, and cyclooxygenase (COX) are reported, accompanied by the activation of the transcriptional activator NF-κB [43,44]. Moreover, increased ROS production causes persistent oxidative damage critical for NF-κB activation and leads to severe cellular injury via generation of reactive species such as PN. Lipids, DNA, and proteins are cellular targets of these free radicals.

Natural products are sources of antioxidant compounds, and there are recent increases in the interest of these compounds due to their activity in many diseases. Curcumin is a popular compound used in traditional Indian medicine. It is soluble in organic solvents, is non-toxic, and is a potent antioxidant owing to the presence of many active functional groups such as betadiketone functional groups, carbon–carbon double bonds, and methoxy and hydroxyl functional substituents in both phenyl rings. The variety of functional groups in curcumin supports its antioxidant attributes and increases the probability of scavenging many types of free radicals. Curcumin increases the activities of antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase [45,46]. Curcumin also inhibits oxygen radical-generating enzymes such as iNOS, xanthine oxidase and COX2. Our results showed that curcumin increases the viability of cells after the immune response induced by LPS+IFN-γ treatment.

Our results are consistent with earlier studies in vitro and in vivo models that confirmed the role of curcumin in protecting from cell death due to increases in free radicals, especially highly oxidizing molecules. Curcumin protects cells against these cytotoxic molecules by scavenging PN and inhibiting iNOS. It also inhibits the LPS-induced immune response in rat vascular smooth muscle cells of through the TLR4-MAPK/NF-κB pathway by ROS-relative [47]. In addition to inhibiting PN formation and NF-κB activation, curcumin inhibits many immune response mediators affected by NF-κB such as cytokines, chemokines, and lipoxigenase. Curcumin also inhibits proliferation various tumor cells, caspase-mediated apoptotic cell death and the
Fig. 3. (A) The inhibitory effects of curcumin on the level of PN released from peroxynitrite donor (SIN-1) in PBS (pH 7.4) with DHR-123 after 2-h incubation. DHR-123 oxidation was significantly reduced by curcumin in a dose-dependent manner compared with SIN-1 alone or SIN-1+DMC or SIN-1 + 1400W. (B) Effect of curcumin on the level of nitrite production from SIN-1. Curcumin and 1400W were incubated with SIN-1 for 2 h. No significant reduction in the amount of nitrite production from SIN-1 was observed in all groups. (C) The inhibitory effects of curcumin and DMC on PN in the presence of DHR-123 after a 15-min direct incubation. Curcumin dose-dependently inhibited PN oxidation of DHR-123, while DMC and 1400W did not inhibit oxidation of DHR-123 by PN. Each value indicates the mean ± S.E.M. of five independent experiments. p ≤ 0.0001 (***)
xanthine/xanthine oxidase system and blocks ROS production in neutrophils. Curcumin inhibits neutrophil activation and infiltration into the inflammatory regions after TPA-induced oxidative stress.

Previous studies confirmed the potent antioxidant activity of curcumin and compared it with that of a structural analogue, DMC, wherein both hydroxyl groups are replaced with methyl groups. Lipid peroxidation was inhibited 82% by curcumin but only 24% by DMC. Inhibition of 1,1-diphenyl-2-picrylhydrazyl (DPPH) by curcumin was 1800 time greater than DMC, confirming the role of the hydroxy group in curcumin as the potent antioxidant source [25]. However, reaction of one-electron with one of phenolic hydroxyl group in curcumin leads to phenoxyl radicals, while in DMC, this is not possible owing to the methylation of the phenolic hydroxyl groups. This shows that the main antioxidant activities of curcumin depend on the presence of phenolic hydroxyl groups and not methyl groups [48].

Our results showed curcumin to be potent in scavenging PN and clearly confirmed the role of hydroxyl groups in scavenging activity through comparison with the a structurally analogous compound, DMC, that did not show any activity against PN. In a previous study, curcumin significantly reduced increased calcium influx and mitochondrial membrane potential loss induced by hydrogen peroxide by inhibiting ROS generation and upregulating antioxidant proteins, including heme oxygenase-1, peroxiredoxin-1, and thioredoxin and antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase [49]. Curcumin has been shown to be a potent inhibitor of cell death via the apoptotic pathway induced by ethanol. Curcumin also significantly enhanced the level of B-cell lymphoma 2 (Bcl-2) protein that regulates the mitochondrial potential, outer mitochondrial membrane, mitochondrial morphology, and the balance between death and survival in neurons and inhibits caspase-3 activation in cortical neurons.

**4. CONCLUSION**

Curcumin has two phenolic hydroxyl groups mediate its potent antioxidant activity, in particular scavenging of PN in vitro. The role of phenolic hydroxyl groups in scavenging PN was clearly shown in different models. Curcumin increases cell viability through scavenging free radicals, including NO and PN that induced cell death during the immune response. Owing to its natural antioxidant activities, scavenging of highly oxidative molecules, lack of toxicity, and rich history in traditional medicine, further study should be devoted to developing curcumin’s antioxidant and pharmacological properties in order to treat diseases related to free radical-induced damage and cell death.

**AVAILABILITY OF DATA AND MATERIALS**

The datasets used and/or analysed during the current study are available from the primary author on reasonable request.

**CONSENT**

It is not applicable.

**ETHICAL APPROVAL**

It is not applicable.

**COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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