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ARTICLE

Evaluation of *Azadirachta indica* Leaf Fractions for in Vitro Antioxidant Potential and Protective Effects against H_2O_2-Induced Oxidative Damage to pBR322 DNA and Red Blood Cells

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We evaluated the protective effects of subfractions of the ethyl acetate fraction (EAF) and the methanolic fraction (MF) from the crude ethanolic extract (CEE) of *Azadirachta indica* A. Juss (neem) leaves against various free radicals and hydrogen peroxide (H_2O_2)-induced oxidative damage to red blood cells (RBCs) and pBR322 DNA. Neem leaf fractions reduced DPPH$^{\bullet}$, ABTS$^{\bullet+}$, superoxide ($O^{\bullet-}$), hydroxyl ($OH^{\bullet}$), and nitric oxide radicals to nonradical forms in a concentration-dependent manner. Treatment with the benzene insoluble fraction from EAF (EBIF), the chloroform insoluble fraction from EAF (ECIF), the chloroform insoluble fraction from MF (MCIF), and the ethyl acetate insoluble fraction from MF (MEIF) significantly mitigated H_2O_2-induced oxidative damage to RBCs and pBR322 DNA. Although we found low in vitro free radical scavenging activity for the benzene insoluble fraction from EAF (EBSF), the chloroform soluble fraction from EAF (ECSF), the chloroform soluble fraction from MF (MCSF), and the ethyl acetate soluble fraction from MF (MESF), these fractions showed no effect on H_2O_2-induced lipid peroxidation and pBR322 DNA damage. High-performance liquid chromatography (HPLC) and TLC-Iatroscan analysis revealed that the greater efficacy of EBIF, ECIF, MCIF, and MEIF may be due to the presence of more polar compounds such as nimbolide and quercetin. Our studies suggest that the antioxidant and protective effects of active neem leaf fractions against H_2O_2-induced lipid peroxidation and pBR322 DNA damage can be attributed to their ability to inhibit various free radicals.

KEYWORDS: Antioxidants; free radicals; H_2O_2; neem leaf fractions; lipid peroxidation; pBR322

1. INTRODUCTION

Living organisms derive energy from oxidation of biomolecules, resulting in the formation of reducing equivalents that flow into the electron transport chain and react with molecular oxygen to form water (*1*). Under certain circumstances, oxygen is also reduced to water via reactive oxygen species (ROS). Superoxide anion ($O_2^{\bullet}$) generated by the transfer of one single free electron to molecular oxygen is converted by dismutation to hydrogen peroxide (H_2O_2) that interacts with Fe^{2+} via the Fenton reaction to form the highly cytotoxic and reactive hydroxyl radical ($OH^{\bullet}$) (*2*, *3*). Although ROS are essential for the organism's vital activities, including phagocytosis, regulation of cell proliferation, intracellular signaling, and synthesis of biologically active compounds and energy, excessive production of ROS causes oxidative stress and chronic diseases such as cardiovascular disease, diabetes, and cancer (*3*−*5*).

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ROS are highly toxic to all types of biomolecules, including proteins, lipids, and DNA. ROS directly react with DNA bases to produce oxidative DNA adducts associated with mutagenesis and carcinogenesis. ROS induce lipid peroxidation of cellular membranes, generating toxic metabolites such as MDA that react with DNA to form adducts. Although all organisms are well-protected against ROS-induced oxidative damage by various enzymatic and nonenzymatic antioxidants as well as DNA repair mechanisms, these protective systems are insufficient to prevent the damage entirely (*4*−*7*). Recently, considerable attention has therefore been focused on identifying antioxidants from natural sources such as functional foods and medicinal plants (*8*, *9*).

Azadirachta indica A. Juss, known in vernacular as neem, is one of the most versatile medicinal plants possessing a wide spectrum of biological activities (*10*). The antioxidative properties of neem leaf extract have been documented both in vitro and in vivo (*11*, *12*). Extensive investigations by us and others have revealed that neem leaf extracts inhibit the development of experimental carcinogenesis by modulating multiple molecular targets in key signaling pathways (*13*−*16*). Recently, we demonstrated the potent in vitro antioxidative and in vivo

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chemopreventive potential of the ethyl acetate fraction (EAF) and the methanolic fraction (MF) obtained from the crude ethanolic extract (CEE) of neem leaf (*17*). This study is a continuation of our efforts aimed at activity-guided fractionation of neem leaf extract and screening for antioxidative potential. To resolve the active fractions from EAF and MF into less polar, intermediate, and more polar compound rich fractions, EAF and MF were further extracted with increasing solvent polarities. Here, we describe the results of our experiments designed to evaluate the protective effects of subfractions of EAF and MF against various free radicals and H_2O_2-induced oxidative damage on pBR322 DNA and red blood cells (RBCs) as well as indentification of the active constituents in the subfractions based on high-performance liquid chromatography (HPLC) and TLC-Iatroscan analysis.

2. MATERIALS AND METHODS

2.1. Chemicals. Ascorbic acid, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), reduced nicotinamide adenine dinucleotide phosphate (NADPH), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), potassium ferricyanide, 2-thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of analytical grade.

2.2. Collection of Plant Material and Preparation of Extract. Fresh mature leaves of *A. indica* collected locally from October to December 2007 were identified by a pharmacognosy expert. The voucher specimens were deposited at the herbarium of the Botany Department, Annamalai University. The leaves of *A. indica* were dried in shade and powdered, and the powder was used for the preparation of the ethanolic extract according to the procedure described by Chattopadhyay (*18*). Air-dried powder (1 kg) of *A. indica* leaves was mixed with 3 L of 70% ethyl alcohol and kept at room temperature for 36 h. The slurry was stirred intermittently for 2 h and left overnight. The mixture was filtered through muslin cloth and the filtrate concentrated under reduced pressure (bath temperature of 40 °C) and dried in a vacuum desiccator. The amount of residue obtained from 1 kg of *A. indica* leaf powder was 48 g. The crude ethanolic extract (CEE) was further fractionated through silica gel (100−200 mesh) column using petroleum ether, ethyl acetate, and methanol. Since the ethyl acetate fraction (EAF) and the methanolic fraction (MF) were shown to possess significant chemopreventive potential against DMBA-induced HBP carcinogenesis, these fractions were used for subfractionation (*17*).

2.3. Extraction of EAF. Extraction of EAF was achieved by solvent partitioning following standard methods described by Marvin and Hewitt (*19*). Eighty grams of EAF was dissolved in 300 mL of an ethyl acetate/water (2/8) mixture. The resulting solution was partitioned three times with benzene (3 × 400 mL) for 24 h at room temperature. Two fractions were obtained: benzene soluble fraction (EBSF) and benzene insoluble fraction (EBIF). These two fractions were dried in a rotary vacuum evaporator. From this, 40 g of EBIF was further dissolved in 200 mL of an ethyl acetate/water (2/8) mixture and further extracted three times with chloroform (3 × 300 mL) for 24 h at room temperature, and two fractions were obtained: chloroform soluble fraction (ECSF) and chloroform insoluble fraction (ECIF). These fractions were dried in a rotary vacuum evaporator. EBSF, EBIF, ECSF, and ECIF were used for testing the antioxidant potential in vitro.

2.4. Extraction of MF. Extraction of MF was achieved by solvent partitioning following standard methods described by Marvin and Hewitt (*19*). Eighty grams of MF was dissolved in 300 mL of a methanol/water (2/8) mixture. The resulting solution was partitioned three times with chloroform (3 × 400 mL) for 24 h at room temperature. Two fractions were obtained: chloroform soluble fraction (MCSF) and chloroform insoluble fraction (MCIF). These fractions were dried in a rotary vacuum evaporator. From this, 40 g of MCIF was further dissolved in 200 mL of a methanol/water (2/8) mixture which was further extracted three times with ethyl acetate (3 × 300 mL) for 24 h at room temperature, and two fractions were obtained: ethyl acetate soluble fraction (MESF) and ethyl acetate insoluble fraction (MEIF). These fractions were dried in a rotary vacuum evaporator. MCSF, MCIF, MESF, and MEIF were used for testing antioxidant potential in vitro.

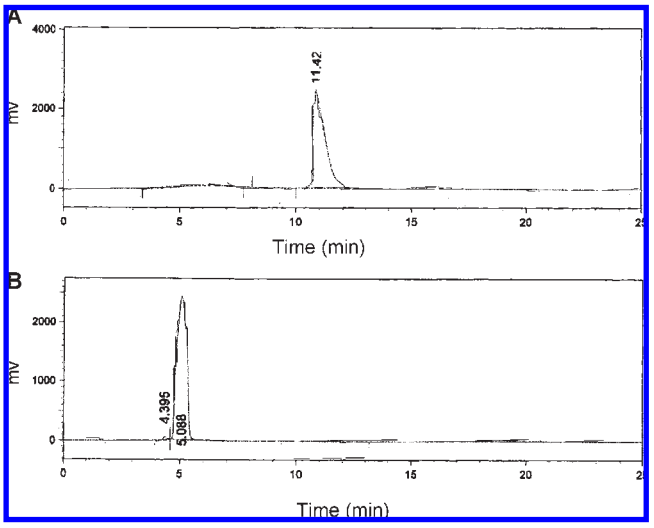

Figure 1. HPLC chromatogram of nimbolide (**A**) and quercetin (**B**).

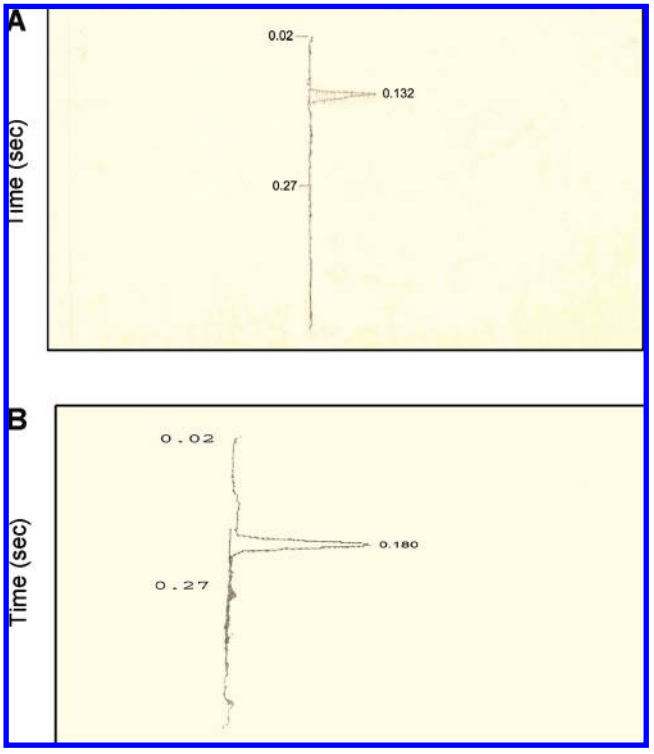

Figure 2. TLC Iatroscan chromatographs of nimbolide (**A**) and quercetin (**B**). The vertical axis represents the distance each peak has moved up the rod, and the horizontal axis is the FID response representing relative areas of each peak.

2.5. HPLC Analysis. A Shimadzu LC-10AT VP HPLC system with an SPD-10A VP PDA detector, a solvent delivery module, and a Rheodyne injector equipped with a 20 μL loop 110 B was used. The neem fractions were dissolved in methanol (20 μg/mL), and 20 μL of the diluted sample was used for injection. The separation was performed on an ODS column (Phenomenes Gemini C_{18} 110A, 5 μm) and elution with an acetonitrile/water (60/40) mixture at a flow rate of 0.5 mL/min. The compounds in subfractions of EAF and MF were identified by comparison with external standard chromatograms of pure nimbolide and quercetin (**Figure 1**).

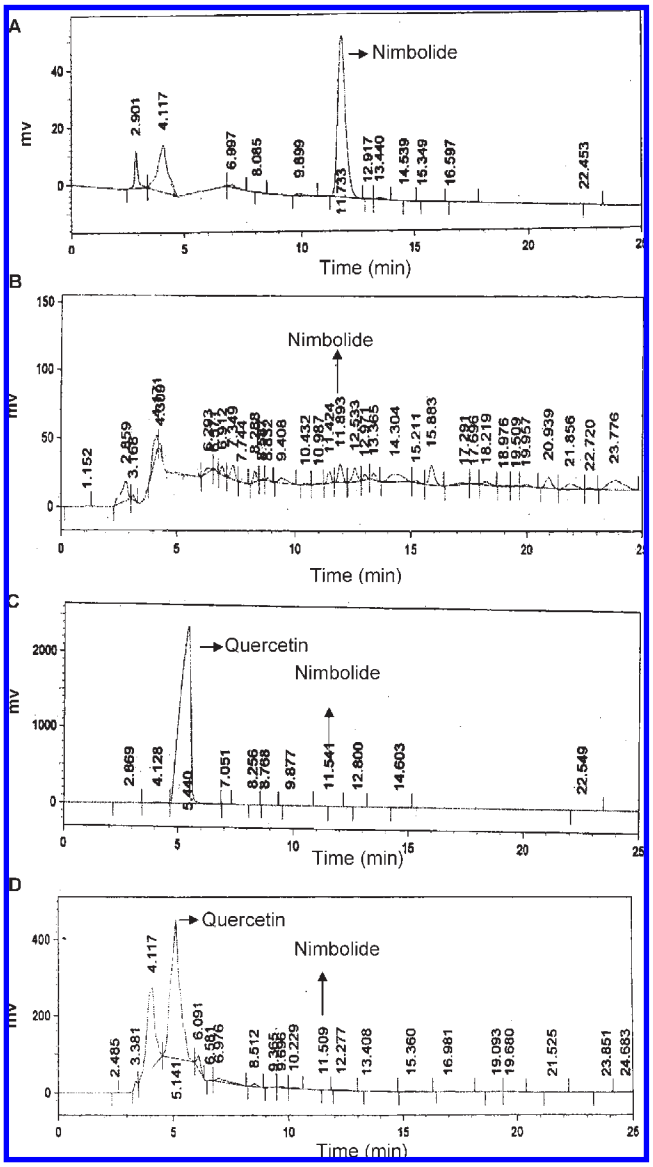

Figure 3. HPLC chromatograms of EBIF (**A**), ECIF (**B**), MCIF (**C**), and MEIF (**D**).

Table 1. Concentrations of Neem Compounds in EBIF, ECIF, MCIF, and MEIF

	concentration (%)	
sample	nimbolide	quercetin
EBIF	2.43	–
ECIF	0.29	–
MCIF	0.05	4.71
MEIF	0.05	4.69

2.6. TLC-Iatroscan Analysis. An Iatroscan TM MK-65 system with a hydrogen-flame ionization detector (FID) was used to identify the compounds in the neem leaf fractions. The instrument was set up according to the manufacturer's specifications. The hydrogen gas flow rate was set to 160 mL/min, and the air flow rate was set to 2.0 mL/min. The flow rates were read from the bottom of the balls located inside the Iatroscan gauges. The chromorods were blank scanned thrice at 30 s per scan to remove contaminants.

Chromorods were placed in the rod holder, cleaned, and activated in the flame of the detector of the Iatroscan instrument. The holder was removed and placed on the spotting guide. A small amount of sample dissolved in methanol (10 mg/mL) was applied to the start using a micropipet. Thin

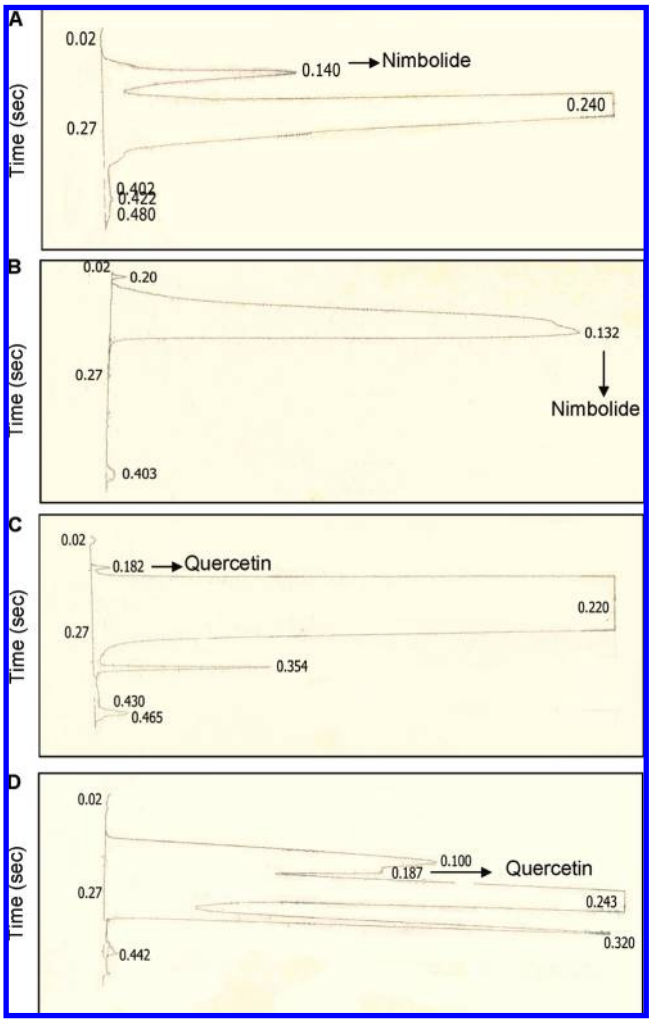

Figure 4. TLC-Iatroscan chromatographs of EBIF (**A**), ECIF (**B**), MCIF (**C**), and MEIF (**D**). The vertical axis represents the distance each peak has moved up the rod, and the horizontal axis is the FID response representing relative areas of each peak.

layers were eluted with an acetonitrile/water (6/4) mixture in the development tank. The rods were dried for few minutes in a drying oven at 60 °C, placed in the sliding frame of the Iatroscan analyzer, and passed through an FID. The individual separated zones were ionized in the hydrogen flame, and the ionization current was amplified and fed into integrates and recorder. The compounds in various fractions were identified by comparison with external standard chromatograms of nimbolide and quercetin (**Figure 2**).

2.7. In Vitro Free Radical Scavenging Assays. The free radical scavenging capacity was evaluated by the DPPH assay described by Blois (*20*). The total antioxidant potential was measured by the ABTS assay that measures the relative ability of antioxidants to scavenge the $ABTS^{\bullet+}$ cation radical generated in the aqueous phase (*21*). Hydroxyl radical scavenging activity was determined by the method of Halliwell et al. (*22*) on the basis of the ability to compete with deoxyribose for hydroxyl radicals. The superoxide anion scavenging activity was determined by the method of Nishimiki et al. (*23*). Superoxide anion derived from dissolved oxygen by a PMS/NADH coupling reaction reduces nitro blue tetrazolium (NBT), which forms a violet-colored complex. The nitric oxide radical inhibition activity was measured by the method of Garrat (*24*) using Griess reagent. The reductive potential was determined according to the method of Oyaizu (*25*) on the basis of the chemical reaction of Fe(III) to Fe(II). Ascorbic acid was used as a positive control.

2.8. Preparation of Erythrocyte Hemolysate and in Vitro Lipid Peroxidation Assay. Blood samples were collected in heparinized tubes,

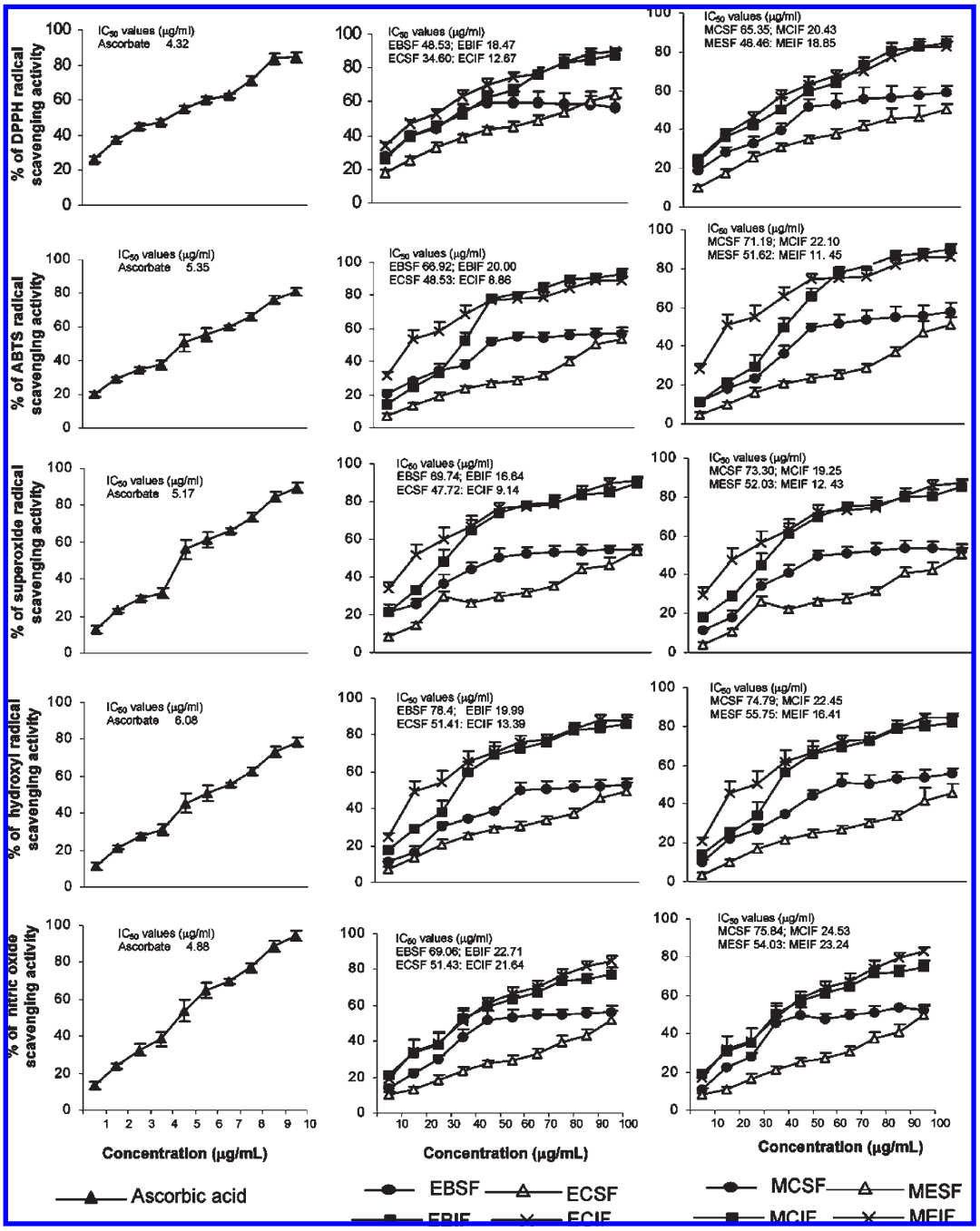

Figure 5. Inhibition of DPPH, ABTS, superoxide, hydroxyl, and nitric oxide radicals by various fractions from EAF and MF. Data are represented as means ± the standard deviation of two independent experiments each performed in triplicate. The percentage of radical scavenging activity = $(Abs_{control} - Abs_{sample})/Abs_{control} \times 100$, where $Abs_{control}$ is the absorbance of the control reaction mixture and Abs_{sample} is the absorbance of the sample of the fractions and standard at different concentrations. IC_{50} values were determined by plotting dose−response curves of radical scavenging activities vs the concentration of fractions using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., San Diego, CA).

and the plasma was separated by centrifugation at 1000*g* for 15 min. After centrifugation, the buffy coat was removed and the packed cells were washed three times with physiological saline. A known volume of erythrocytes was lysed with hypotonic phosphate buffer (pH 7.4). The hemolysate was separated by centrifugation at 2500*g* for 15 min at 2 °C and used for the in vitro lipid peroxidation assay by the method of Cynamon et al. (*26*). Malondialdehyde, a product of PUFA peroxidation, was assessed as the thiobarbituric acid derivative following incubation of erythrocytes with H_2O_2 and neem leaf fractions.

2.9. Assessment of DNA Protection by Neem Leaf Fractions. Approximately 2 μg of pBR322 DNA, suspended in 10 μL of 50 mM sodium phosphate buffer (pH 7.4), was pretreated with different concentrations of neem leaf subfractions (2−12 μg/mL) for 30 min followed by incubation with 100 mM H_2O_2, 27.10 mM EDTA-Na_2, and 8.70 mM $FeSO_4$ for 1 h (*27*). Following incubation, the supercoiled and open circular forms of DNA were loaded onto a 1% agarose gel containing ethidium bromide, electrophoresed in 1× Tris acetate/EDTA buffer for 1 h at 75 V, and photographed under transillumination. H_2O_2-induced damage was assessed as an increase in level of the open circular form of DNA, and protection of pBR322 was assessed as a decrease in the level of the open circular form.

2.10. Statistical Analysis. The data are expressed as means ± the standard deviation (SD). The IC_{50} for in vitro antioxidant potential was calculated using linear regression analysis. Data for reducing power and in vitro lipid peroxidation were statistically analyzed using analysis of variance (ANOVA), and the group means were compared by the least

significant difference test (LSD). The results were considered statistically significant if the $p < 0.05$.

3. RESULTS

3.1. HPLC Analysis for Assessing Neem Compounds in Subfractions of EAF and MF. HPLC analysis was conducted to identify the components in the neem leaf fractions. The HPLC chromatograms are shown in **Figure 3** and the neem constituents found in EBIF, ECIF, MCIF, and MEIF presented in **Table 1**. The results reveal that EBIF and ECIF contain nimbolide with retention times of 11.73 and 11.89 min, respectively. HPLC analysis of MCIF and MEIF resulted in the identification of nimbolide and quercetin with retention times of 11.54 and 11.50 min and 5.44 and 5.141 min, respectively. These two compounds were not identified in the rest of the fractions.

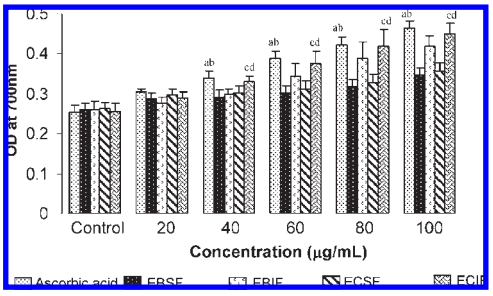

Figure 6. Reducing potentials of ascorbic acid, EBSF, EBIF, ECSF, and ECIF. The letter a indicates a value significantly different from those of EBSF and ECSF ($p < 0.001$) (ANOVA followed by LSD), the letter b a value significantly different from those of EBIF and ECIF ($p < 0.01$), the letter c a value significantly different from those of EBSF and ECSF ($p < 0.01$), and the letter d a value significantly different from that of EBIF ($p < 0.01$).

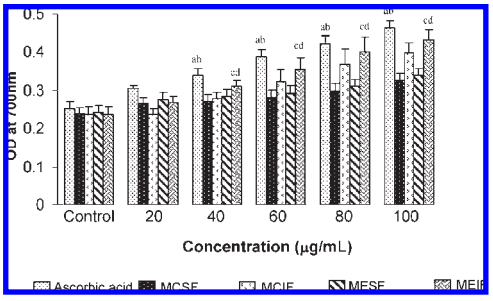

Figure 7. Reducing potentials of ascorbic acid, MCSF, MCIF, MESF, and MEIF. The letter a indicates a value significantly different from those of MCSF and MESF ($p < 0.001$) (ANOVA followed by LSD), the letter b a value significantly different from those of MCIF and MEIF ($p < 0.01$), the letter c a value significantly different from those of MCSF and MESF ($p < 0.01$), and the letter d a value significantly different from that of MEIF ($p < 0.01$).

3.2. TLC Analysis for Assessing Neem Compounds in Subfractions from EAF and MF. TLC-Iatroscan was used to identify the components in different fractions from EAF and MF. Chromatograms of neem leaf fractions are presented in **Figure 4**. The results reveal that EBIF and ECIF contain nimbolide with retention times 0.140 and 0.132 s, respectively. The active compound in MCIF and MEIF was identified as quercetin (times of 0.182 and 0.187 s, respectively). The rest of the fractions did not contain either nimbolide or quercetin.

3.3. In Vitro Antioxidant Assays. Figure 5 shows the free radical scavenging effects of ascorbic acid and various fractions from EAF and MF in vitro and their IC_{50} values. Analysis of the free radical scavenging activities of the fractions revealed a concentration-dependent antiradical activity resulting from reduction of $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O^{\bullet-}$, $OH^{\bullet}$, and nitric oxide radicals to their nonradical forms. The scavenging activity of ascorbic acid, a known antioxidant used as a positive control, was however higher compared to that of the neem leaf fractions. **Figures 6** and **7** present the reduction potentials of various fractions. The reducing power of ascorbic acid and fractions increased gradually with an increase in concentration. Of the various fractions analyzed, EBIF, ECIF, MCIF, and MEIF exhibited the maximum effect.

3.4. In Vitro Lipid Peroxidation Assay. Table 2 shows the effect of EAF and MF subfractions on H_2O_2-induced lipid peroxidation on erythrocytes. The level of TBARS was significantly increased in cells treated with H_2O_2 compared to untreated cells. Treatment with EBIF, ECIF, MCIF, and MEIF significantly decreased the levels of TBARS compared with that of H_2O_2-treated cells, and the effect was more pronounced at 12 μg/mL for EBIF and ECIF and at 16 μg/mL for MCIF and MEIF. No significant changes were observed between untreated cells and cells treated with neem leaf fractions alone. No effect was observed in EBSF, ECSF, MCSF, or MESF.

3.5. Assessment of DNA Protection by Neem Leaf Fractions. Figure 8 shows the agarose gel electrophoresis of pBR322 DNA treated with 100 mM H_2O_2 in the presence and absence of different concentrations of neem leaf fractions. As compared to the control and plasmid DNA treated with fractions alone (lanes 1 and 2), plasmid DNA treated with H_2O_2 (lane 3) increased the relative intensity of the band corresponding to open circular forms of DNA. Addition of neem leaf fractions EBIF, ECIF, MCIF, and MEIF in increasing concentrations (lanes 4−9) reduced the intensity of the bands corresponding to the open

Table 2. Effects of EBSF, EBIF, ECSF, ECIF, MCSF, MCIF, MESF, and MEIF on H_2O_2-Induced Lipid Peroxidation on Erythrocytes

sample	untreated RBCs	RBCs with neem leaf fractions (20 μg/mL)	RBCs with H_2O_2 (3%)	RBCs with H_2O_2 (3%) and neem leaf fractions (4 μg/mL)	RBCs with H_2O_2 (3%) and neem leaf fractions (8 μg/mL)	RBCs with H_2O_2 (3%) and neem leaf fractions (12 μg/mL)	RBCs with H_2O_2 (3%) and neem leaf fractions (16 μg/mL)	RBCs with H_2O_2 (3%) and neem leaf fractions (20 μg/mL)
EBSF	3.21 ± 0.30	3.12 ± 0.23	5.09 ± 0.42^a	5.00 ± 0.38	4.92 ± 0.26	4.96 ± 0.27	5.08 ± 0.52	4.92 ± 0.52
EBIF	3.21 ± 0.30	3.12 ± 0.23	5.09 ± 0.42^a	4.82 ± 0.38^b	4.41 ± 0.23^c	3.52 ± 0.19^d	3.71 ± 0.26^d	4.12 ± 0.32^c
ECSF	3.21 ± 0.30	3.12 ± 0.23	5.09 ± 0.42^a	4.93 ± 0.28	4.94 ± 0.31	4.86 ± 0.44	4.89 ± 0.46	4.57 ± 0.12
ECIF	3.21 ± 0.30	3.12 ± 0.23	5.09 ± 0.42^a	4.70 ± 0.43^b	4.26 ± 0.37^c	3.56 ± 0.26^d	3.68 ± 0.32^d	3.89 ± 0.28^c
MCSF	3.36 ± 0.36	3.30 ± 0.28	4.98 ± 0.39^a	4.97 ± 0.52	4.95 ± 0.38	4.90 ± 0.41	4.86 ± 0.44	4.88 ± 0.53
MCIF	3.36 ± 0.36	3.30 ± 0.28	4.98 ± 0.39^a	4.92 ± 0.42	4.86 ± 0.38^b	4.72 ± 0.28^b	4.10 ± 0.39^c	4.62 ± 0.50^b
MESF	3.36 ± 0.36	3.30 ± 0.28	4.98 ± 0.39^a	4.93 ± 0.58	4.91 ± 0.38	4.80 ± 0.26	4.82 ± 0.28	4.83 ± 0.52
MEIF	3.36 ± 0.36	3.30 ± 0.28	4.98 ± 0.39^a	4.96 ± 0.29	4.40 ± 0.36^b	4.00 ± 0.41^c	3.86 ± 0.28^d	4.28 ± 0.60^b

[a] Significantly different from the value of untreated cells [ANOVA followed by LSD ($p < 0.001$)]. [b] Significantly different from the value of H_2O-treated cells [ANOVA followed by LSD ($p < 0.05$)]. [c] Significantly different from the value of H_2O-treated cells [ANOVA followed by LSD ($p < 0.01$)]. [d] Significantly different from the value of H_2O-treated cells [ANOVA followed by LSD ($p < 0.001$)].

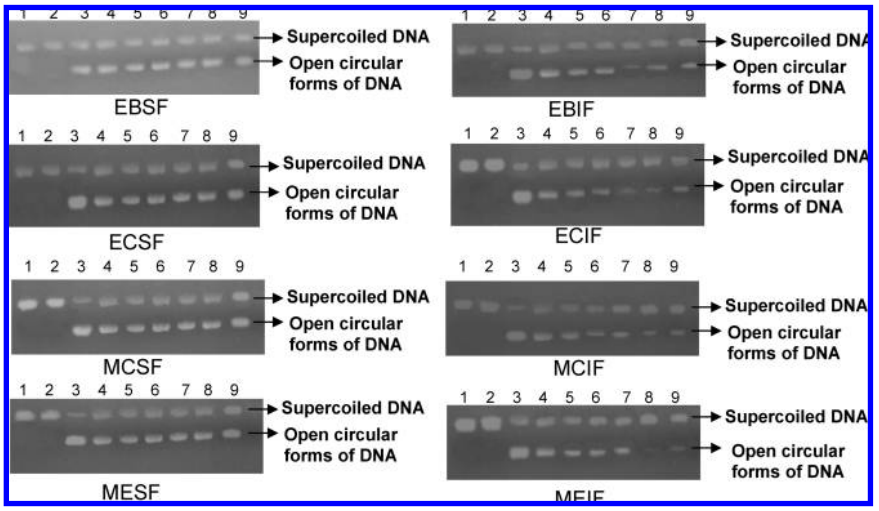

Figure 8. Agarose gel electrophoresis of pBR322 DNA treated with 100 mM H_2O_2 in the presence and absence of different concentrations of various fractions from EAF and MF. pBR322 DNA (2 μg) in 50 μL of buffer was pretreated with different concentrations of neem leaf subfractions (2−12 μg/mL) prior to treatment with 100 mM H_2O_2 for 30 min followed by incubation with 100 mM H_2O_2, 27.10 mM EDTA-Na_2, and 8.70 mM $FeSO_4$ for 1 h. Electrophoresis was conducted in a 1% agarose gel. H_2O_2-induced damage was assessed as an increase in the level of the open circular form of DNA and protection of pBR322 by a decrease in the level of the open circular form: lane 1, pBR322 DNA; lane 2, pBR322 with neem leaf fraction alone; lane 3, pBR322 with 100 mM H_2O_2; lane 4, pBR322 with 100 mM H_2O_2 and 2 μg/mL neem leaf fraction; lane 5, pBR322 with 100 mM H_2O_2 and 4 μg/mL neem leaf fraction; lane 6, pBR322 with 100 mM H_2O_2 and 6 μg/mL neem leaf fraction; lane 7, pBR322 with 100 mM H_2O_2 and 8 μg/mL neem leaf fraction; lane 8, pBR322 with 100 mM H_2O_2 and 10 μg/mL neem leaf fraction; and lane 9, pBR322 with 100 mM H_2O_2 and 12 μg/mL neem leaf fraction.

circular form, and the effect was more pronounced at 8 μg/mL for EBIF and ECIF and at 10 μg/mL for MCIF and MEIF. No differences in band detection were observed between control DNA and DNA treated with neem leaf fractions alone. No effect was observed in EBSF, ECSF, MCSF, and MESF.

4. DISCUSSION

In this study, neem leaf fractions exhibited a concentration-dependent antiradical activity resulting from reduction of $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O^{\bullet-}$, $OH^{\bullet}$, and nitric oxide radicals to their nonradical forms. $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals accept an electron or hydrogen from compounds in the neem leaf fractions to become stable nonradical forms. The hydrogen donating ability of neem leaf fractions was further supported by its reduction potential and strengthens its potential antioxidant activity. Under certain conditions, O_2 is reduced to H_2O via $O^{\bullet-}$ and H_2O_2 that favors the formation of other reactive oxygen ($OH^{\bullet}$) and nitrogen ($ONOO^{-}$) species (*4*, *5*). In particular, H_2O_2 can be decomposed by the Fe^{3+}–EDTA complex via the Fenton reaction to produce hydroxyl radicals that are recognized to induce lipid peroxidation and DNA strand breaks (*28*−*30*). Thus, excessive production of toxic radical species is recognized to cause deleterious changes in DNA, lipid, and protein oxidation (*4*, *5*). This study demonstrates that the ability of neem leaf fractions to quench $O^{\bullet-}$, $OH^{\bullet}$, and nitric oxide radicals is directly linked to the prevention of the process of propagation of lipid peroxidation and DNA damage associated with carcinogenesis. This mechanism is also further supported by the protective effects of neem leaf fractions against H_2O_2-induced lipid peroxidation in the RBCs and strand breaks in pBR322 DNA that may be attributed to the potent antioxidant activity as evidenced by in vitro radical scavenging and reducing potential. Furthermore, the active constituents in the neem leaf fractions may also possibly interact directly with DNA and protect pBR322 DNA from hydroxyl radical-induced strand breaks. Our results are in line with the free radical scavenging effects of neem leaf extracts and neem compounds reported in the literature (*11*, *17*, *31*, *32*). Recently, we have documented the free radical scavenging potential of nimbolide against various free radicals (*33*). Quercetin was found to exhibit superoxide and nitric oxide radical scavenging effects (*34*, *35*). Morales et al. (*36*) have demonstrated that quercetin treatment prevents cadmium-induced renal tubular damage and oxidative stress by decreasing the extent of lipid peroxidation and enhancing the antioxidant status.

Compared with other fractions, EBIF, ECIF, MCIF, and MEIF exhibited a greater inhibitory effect on $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O^{\bullet-}$, $OH^{\bullet}$, and nitric oxide radicals, H_2O_2-induced lipid peroxidation, and pBR322 DNA damage. Although we found low in vitro free radical scavenging activity for EBSF, ECSF, MCSF, and MESF, these fractions showed no effect on H_2O_2-induced lipid peroxidation and pBR322 DNA damage. This may be attributed to the retention of intermediate or less polar compounds. The antioxidant potential of active neem leaf fractions may be ascribed to the presence of more polar compounds. HPLC and TLC-Iatroscan analysis revealed that EBIF and ECIF contain nimbolide while MCIF and MEIF were rich in nimbolide and quercetin. While the antioxidant effects of nimbolide may be due to the α,β-unsaturated ketone element, ester groups, and ester derivatives, those of quercetin have been ascribed to the hydroxyl groups (*33*, *37*). Taken together, our studies suggest that neem leaf contains antioxidant phytochemicals that could be therapeutically beneficial. The results of this study also provide evidence that the antioxidant and protective effects of neem leaf fractions against H_2O_2-induced lipid peroxidation and pBR322 DNA damage can be attributed to their ability to inhibit various free radicals. If these observations can be extrapolated to in vivo systems, the radical scavenging ability of neem leaf fractions may also possibly contribute to its protective effects against free radical-induced oxidative stress and carcinogenesis. Therefore, further studies are required to establish its in vivo antioxidant and anticarcinogenic effects using different experimental animal models.

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