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Research Article

Phytochemical Analysis, *In Vitro* Free Radical Scavenging, and LDL Protective Effects of Different Solvent Fractions of *Calotropis procera* (R.) Br. Root Bark Extract

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In the present study, the methanolic extract of *Calotropis procera* root bark was subjected to solvent-solvent partitioning using n-hexane fraction (HF), dichloromethane fraction (DMF), ethyl acetate fraction (EAF), and methanol fraction (MF). The resultant fractions were tested for antioxidant activity using *in vitro* radical scavenging assay. Preliminary phytochemical investigation revealed the presence of varying proportions of secondary metabolites in solvent fractions such as glycosides, flavonoids, triterpenoids, sterols, and polyphenolic compounds. The total phenolic content of EAF was 25.7 ± 3.12 mg TAE/g followed by 19.05 ± 3.29 mg TAE/g in DMF. The total flavonoid content was 13.69 ± 1.74 mg QUE/g in DMF and 11.4 ± 1.88 mg QUE/g in EAF. The EAF showed significant radical-scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH, IC50 = 369.87 μg/mL), nitric oxide (IC50 = 317.46 μg/mL), hydrogen peroxide (H2O2 , IC50 = 396.85 μg/mL), and hydroxyl radicals (IC50 = 195.39 μg/mL). DMF was most effective in scavenging superoxide radicals (IC_{50} = 679.60 μg/mL), while greater metal chelating activity was exhibited by MF (IC_{50} = 614.73 μg/mL). Moreover, the total antioxidant activity for EAF was found to be 94.14 ± 9.114 mg AAE followed by DMF (68.10 ± 8.78 mg AAE). EAF also significantly reduced the formation of thiobarbituric acid reactive substances in a dose-dependent manner in $CuSO_4$. The observed antioxidant effect might be attributed to the presence of secondary metabolites. Subsequent GC-MS analysis of EAF confirmed the presence of lupeol, α-amyrin, β-amyrin, and ursolic acid. The current investigation reveals that the high polyphenolic and antioxidant pentacyclic triterpenes in EAF of *C. procera* root bark methanol extract correlates with its good antioxidant activity and can provide protection against free radicals-induced damage in a variety of chronic health conditions.

1. Introduction

Excessive oxidative stress plays a crucial role in cellular damage and apoptosis and is recognized as a major contributor to the pathogenesis of chronic diseases such as cancer [1], stroke [2], diabetes, cardiovascular diseases [3, 4], atherosclerosis, chronic obstructive pulmonary disease, Alzheimer's, and other neurodegenerative diseases [5]. It is

thought that numerous malignancies arise due to the interplay of free radicals and DNA, leading to genetic alterations that impede the process of cell division [6].

Overproduction of ROS causes structural modifications of cellular proteins and alters their functions, which leads to cellular dysfunction and disruption of essential cellular processes [7, 8]. A variety of mechanisms are involved in ROS-induced protein damage, including site-specific amino acid modifications, electric charge alteration, peptide chain fragmentation, aggregation of cross-linked products, enzyme inactivation, and proteolysis susceptibility [9, 10].

Phenolics are one of the diverse arrays of phytochemicals occurring ubiquitously in food and medicinal plants as secondary metabolites. The implication of reactive oxygen species in a wide variety of pathobiological manifestations and the beneficial role of polyphenols as potential natural antioxidants have been extensively studied and emphasized in previously published reports [11, 12]. Antioxidant phytochemicals, which are present in a variety of foods and medicinal plants, are crucial for the prevention and management of chronic diseases caused by oxidative stress. They are reported to have potent anti-inflammatory, antioxidant, and free radical scavenging properties, which also form the basis for additional biological activities and therapeutic benefits such as protection against cancer, cardiovascular disease, diabetes mellitus, obesity, and neurodegenerative diseases [13, 14]. They either function by scavenging reactive oxygen species or by defending the body's endogenous antioxidative defense processes [15]. It has been documented that the antioxidant activity of phenolic compounds is mainly due to their redox characteristics, hydrogen-donating, singlet oxygen quenching, chain breaking, and metal chelating capabilities [16, 17].

There is still a growing need to identify plant species that have antioxidant potential and therapeutic usefulness. *Calotropis procera* (Ait.) R. Br. is a xerophytic shrub, belonging to the Asclepiadaecae family and has a wide presence throughout Asia and Africa. The significant therapeutic potential of the different parts of the plant has been reported. Traditionally, the latex is used to treat vertigo, alopecia, hair loss, toothache, intermittent fevers, joint inflammation, and paralysis, while the powdered root is used to treat helminthiasis, bronchitis, asthma, leprosy, dermatitis, and elephantiasis. The leaves are used to relieve joint discomfort and reduce swelling [18].

The medicinal values of this plant have been recognized in light of scientific studies conducted to establish the credentials of *C. procera* as a valuable alternative system of medicine. Different parts of *C. Procera* have shown diverse pharmacological activities including antiinflammatory, analgesic, antidiarrheal, hepatoprotective, anticonvulsant, antiulcer, antitumor, antidiabetic, antifertility, antimicrobial, antimalarial, anthelmintic, insecticidal, and spasmolytic activities [19, 20]. Researchers have reported the presence of pentacyclic triterpenes α-amyrin, β-amyrin, β-sitosterol, stigmasterol, and lupeol in *C. procera* root bark [19, 21, 22]. Pentacyclic triterpenes possess antioxidant properties and exert a protective effect against diabetes, hyperlipidemia and atherosclerosis, cardiovascular diseases, and cancer [23, 24].

Therefore, the present investigation was aimed at phytochemical analysis and evaluation of *in vitro* antioxidant and LDL protective efficacy of *C. procera* (Ait.) R. Br. root bark methanol crude extract and its different solvent fractions.

2. Materials and Methods

2.1. Materials and Instruments. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), quercetin dihydrate, L-ascorbic acid and bovine serum albumin were purchased from Himedia Laboratories (Mumbai, India). Folin–Ciocalteu's phenol (FCP) reagent, 2-thiobarbituric acid (TBA), trichloroacetic acid, and butylated hydroxytoluene (BHT) were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India), Heparen 5000IU Injection (Claris Lifesciences Ltd, India) was purchased from commercial source. All chemicals and reagents used were of analytical grade and procured from approved chemical suppliers. Equipment such as a cooling centrifuge (Remi Instruments Division, Vasai, India) and UV-visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, USA) was used in the study.

2.2. Plant Collection and Processing. Roots of *C. procera* (Ait.) R. Br. were collected from the bank of Bhadar River in Dhoraji (21° 45.2724′N, 70° 25.1586′E), Gujarat, India. The taxonomic status of the plant was verified by Dr. Rajesh Raviya, Professor of Botany, Department of Biology, MVM Science and Home Science College, Rajkot, India. The roots were washed with water and the bark was peeled off using a knife and air dried for 10 days. The dried plant material was pulverized into powder and stored in an air-tight container till further usage.

2.3. Extraction and Fractionation Method. *C. procera* root bark (100 g) was thoroughly extracted with methanol in a Soxhlet apparatus for 72 h at a regulated temperature (40°C). The final product was filtered using Whatman filter paper (No. 42). The resulting filtrate was concentrated at a low temperature in a water bath (40°C) to obtain *C. procera* root bark methanol crude extract (CPRME). The crude extract was then subjected to solvent-solvent fractionation following the Kupchan method modified by VanWagenen et al. [25]. Briefly, the dried crude extract (20 g) was subjected to solvent-solvent partitioning with solvents of increasing polarity: n-hexane, dichloromethane, ethyl acetate, and methanol (3×200 mL for each solvent type) in the darkness. All fractions were evaporated to dryness at a low temperature of 40°C to yield hexane fraction (HF), dichloromethane fraction (DMF), ethyl acetate fraction (EAF), and methanol fraction (MF), respectively. At each

step of solvent fractionation, 40 mL of distilled water was added. Methanol insoluble residues were considered as an aqueous fraction (AF). Fractions were stored in an air-tight container until further use.

2.4. Preliminary Phytochemical Investigation. CPRME and solvent fractions were subjected to qualitative phytochemical screening for the presence of various secondary metabolites [26].

2.5. Qualitative TLC Fingerprinting. Crude extract/fractions (10 mg/mL) were prepared and filtered through Whatman I filter paper. 5.0 μL of extract/fractions were applied as a thin band on 10 cm × 8 cm Silica gel 60 F254 TLC plate (Merck, Germany). The plate was developed in a glass chamber presaturated with toluene: ethyl acetate: glacial acetic acid (8 : 2 : 0.5) for 20 min. After visualization of fluorescent bands under UV light, the plate was sprayed with freshly prepared anisaldehyde-sulfuric acid reagent and placed in an oven at 110°C for 5 min for the development of color bands.

2.6. Total Phenolic Content (TPC). The quantification of total phenolic content in the crude extract and fractions was conducted using the modified Folin–Ciocalteu method as described by Wolfe and colleagues [27]. An aliquot of the crude extract/fractions (1 mg/mL) was mixed with 2 mL Folin–Ciocalteu reagent (diluted 1 : 10 v/v with water) and 2 mL of sodium carbonate (Na_2CO_3, 75 g/L). The reaction mixture was vortexed for 15 s and allowed to stand in darkness for 30 min at 25°C for color development. A standard curve was plotted using different concentrations of tannic acid as a reference standard (10, 20, 30, 40, and 500 μg/mL). Absorbance was then measured at 760 nm using a UV spectrophotometer. Total phenolic content was expressed in terms of tannic acid equivalent (TAE/g dry extract or fraction).

2.7. Total Flavonoid Content (TFC). The method described by Ordoñez et al. [28] was used to determine the total flavonoid content. To 0.5 mL of crude extract/fractions (1 mg/mL), 1.5 mL of methanol, 100 μL of aluminum chloride ($AlCl_3$, 10% w/v), 100 μL of 1 M potassium acetate, and 2.8 mL of distilled water were added. After 1.5 h of incubation at room temperature, the absorbance was measured at 420 nm. A standard curve was plotted by preparing different concentrations of quercetin in methanol as a reference standard (20, 40, 60, 80, and 100 μg/mL) and total flavonoid content were expressed in terms of mg of quercetin equivalent (QUE/g dry extract or fraction).

2.8. DPPH Radical Scavenging Activity. For *in vitro* antioxidant study, a range of concentrations of crude extracts and fractions (100, 200, 300, 600, and 1000 μg/mL) were used based on trial and error. Ascorbic acid is used as standard in all tests. The free radical scavenging ability of crude extract/fractions was assessed by DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay (DRSA) as described by Choi et al. [29]. 1.6 mL of different concentrations of crude extract/fractions (100, 200, 300, 600, and 1000 μg/mL) was mixed with 2.4 mL of methanolic DPPH solution (0.1 mM). The test tube contents were mixed vigorously and left at room temperature for 30 min in the dark. The absorbance was determined spectrophotometrically at 517 nm. Ascorbic acid served as the standard reference and 10, 25, 50, 100, and 200 μg/mL concentrations were used to plot the standard curve. Percentage DPPH radical scavenging activity (% DRSA) as evidenced by discoloration of DPPH was calculated by the following equation:

$$\%\text{DRSA} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100, \quad (1)$$

where A_1 is the absorbance of the sample (extract/fractions/standard), while A_0 is the absorbance of the control.

2.9. Nitric Oxide ($NO^{\bullet}$) Radical Scavenging Activity. Nitric oxide radicals were produced from sodium nitroprusside solution at physiological pH [30]. 4 mL sodium nitroprusside (10 mM) was mixed with 1 mL of the test extract/fractions (50, 100, 200, 400, and 600 μg/mL) in PBS (pH 7.4). The mixture was incubated at 25°C for 150 min. An aliquot of incubation mixture (0.5 mL) was mixed with 1 mL of 1% sulphanilic acid reagent (in 2% orthophosphoric acid). To this, 1 mL of 0.1% naphthylethylenediamine (NEDA) was added and allowed to stand for 30 min in the dark to complete the diazotization process. The same process was carried out with ascorbic acid at 25, 50, 100, 150, and 200 μg/mL concentrations. The intensity of a pink chromophore was recorded at 540 nm against the corresponding blank solution with PBS in place of the sample. The % of nitric oxide radical scavenging was calculated using the following equation:

$$\%\ \text{Scavenging} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100, \quad (2)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the crude extract/fraction/standard.

2.10. Hydrogen Peroxide (H_2O_2) Scavenging Activity. Hydrogen peroxide scavenging assay was conducted following the method described by Ruch et al. [31]. To 1 mL crude extract/fractions (50, 100, 200, 400, and 600 μg/mL) and ascorbic acid (10, 25, 50, 100, and 200 μg/mL), 3.4 mL phosphate buffer was added and mixed. The reaction was initiated by adding 0.6 mL of 43 mM H_2O_2 solution and vortexed. The absorbance of the reaction mixture was recorded at 230 nm after 10 min, against a blank. A blank solution contained only phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging by the extract/fractions and the standard was calculated using the following equation:

$$\% \text{ Scavenging} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100, \quad (3)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the crude extract/fraction/standard.

2.11. Hydroxyl ($OH^{\bullet}$) Radical Scavenging Activity. The scavenging ability of crude extract/fractions on hydroxyl radicals was performed according to Smirnoff and Cumbes method [32]. Crude extract/fractions at different concentrations (50, 100, 200, 400, and 800 μg/mL) were added to the 1 mL reaction mixture consisting of 1.5 mM $FeSO_4$ and 0.3 mL of sodium salicylate (20 mM). The reaction was started by adding 0.7 mL of H_2O_2 (6 mM), followed by incubation at 37°C for an hour. The absorbance of hydroxylated salicylates was measured at 562 nm. The same procedure was carried out with ascorbic acid at 25, 50, 100, 200, and 300 μg/mL concentrations. The % of scavenging ability was calculated using the following formula:

$$\% \text{ Inhibition} = \left[\frac{(A_0 - (A_1 - A_2))}{A_0}\right] \times 100, \quad (4)$$

where A_0 = absorbance without sample, A_1 = absorbance with sample, and A_2 = absorbance of sample omitting sodium salicylate.

2.12. Superoxide ($^{\bullet}O2^{-}$) Radical Scavenging Activity. Superoxide radical scavenging activity of crude extract/fractions was performed according to the method by Beauchamp and Fridovich [33] with some modifications. Various concentrations of crude extract/fractions (100, 200, 300, 600, and 1000 μg/mL) were added to the reaction mixture containing 100 μL EDTA (0.1 M), 200 μL sodium cyanide (NaCN, 0.0015%), 50 μL riboflavin (0.12 mM), 100 μL nitroblue tetrazolium (NBT, 1.5 mM), and phosphate buffer (67 mM, pH 7.8) keeping the total volume up to 3 mL. After 15 min of consistent illumination, the optical density of the mixture at 530 nm was measured. Ascorbic acid at 25, 50, 100, 200, and 300 μg/mL was used as a reference standard. A parallel blank in the identical conditions was run with distilled water in place of the sample in the reaction mixture. The percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \left[\frac{(A_0 - (A_1 - A_2))}{A_0}\right] \times 100, \quad (5)$$

where A_0 = absorbance without sample, A_1 = absorbance with sample, and A_2 = absorbance of sample omitting NBT.

2.13. Metal Ion Chelating Activity. The ability of the crude extract/fractions to chelate iron ions was estimated as per Gülçin method [34]. Different concentrations of crude extract/fractions (100, 200, 300, 600, and 1000 μg/mL) and EDTA as standard (25, 50, 100, 200, and 300 μg/mL) were added to 2.5 mL of 2 mM $FeCl_3$. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine (final volume adjusted to 4 mL with methanol), mixed thoroughly, and allowed to stand at room temperature for 10 min. The absorbance of the color produced was measured at 562 nm. The percentage of inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{[A_0 - (A_1 - A_2)]}{A_0} \times 100, \quad (6)$$

where A_0 = absorbance of the control, containing $FeCl_3$ and ferrozine only, A_1 = absorbance in the presence of the tested samples, and A_2 = absorbance of the sample under identical conditions as A_1 with methanol instead of $FeCl_3$ solution.

2.14. Total Antioxidant Capacity (TAC). The TAC of crude extract/fractions was estimated as per the method reported by Prieto et al. [35] with some modifications. An aliquot (0.5 mL) of crude extract/fractions (1 mg/mL) was mixed with 3 mL of the reaction mixture containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 1% ammonium molybdate. The tubes were incubated at 95°C for 10 min and the optical density was recorded at 695 nm using a spectrophotometer against blank after cooling at room temperature. The same approach was used to plot an ascorbic acid standard curve at various concentrations (25, 50, 100, 200, and 300 μg/mL). The results were expressed as mg ascorbic acid equivalents (AAE/g dry extract or fraction).

2.15. Reducing Power. The reducing power of crude extract/fractions was determined according to the method described by Oyaizu [36] with some modifications. To 2.5 mL extracts/fractions of various concentrations (100, 200, 400, 800, and 1200 μg/mL) and ascorbic acid (25, 50, 100, 200, and 300 μg/mL), 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide ($K_3Fe(CN)_6$, 1%, w/v) were mixed. The resultant mixture was incubated at 50°C for 20 min. The reaction was then stopped by adding 2.5 mL of 10% of TCA solution and then centrifuged at 3000 rpm for 10 min supernatant was collected. 2.5 mL of supernatant, 2.5 mL of distilled water, and 0.5 mL of ferric chloride solution (0.1%, w/v) were mixed thoroughly. The absorbance of the greenish-blue chromogen was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. A parallel blank was run replacing the sample with 2.5 mL distilled water.

2.16. Lipid Peroxidation

2.16.1. Isolation of Human Serum LDL. LDL was isolated using a heparin-citrate buffer precipitation method developed by Wieland and Seidel [37]. The hyperlipidemic plasma was vortexed with 50 mL of heparin-citrate buffer (prepared by adding 5000 IU/L heparin to 100 mL 0.064 M trisodium citrate, pH 5.05 adjusted with 5 M HCl) and incubated for 10 minutes at room temperature. The white precipitates were centrifuged at 3500 rpm at 4°C and resuspended in 1 mL of phosphate-buffered saline (PBS,

pH 7.4). LDL-C protein content was determined using the Lowry method modified by Pomory using bovine serum albumin as standard [38].

2.16.2. Induction of LDL Oxidation. In this study, LDL oxidation was performed according to the method described by Chumark et al. [39]. An aliquot of LDL suspension (containing 200 μg LDL) in 10 mM phosphate buffered saline (PBS, pH 7.4) was incubated with 100 μL of different concentrations (25, 50, 100, 200, 500, 1000, and 1500 μg/mL) of CPRME crude extract and its solvent fractions in a total volume of 1.5 mL for 30 min at 37°C. LDL oxidation was initiated by adding 10 μL of freshly prepared 0.167 mM $CuSO_4$ to all tubes and incubating again for 6 h. At the end of the incubation period, oxidation kinetic was terminated by adding 10 μL EDTA (10 mM).

2.16.3. Measurement of Thiobarbituric Acid Reactive Substance (TBARS) in LDL. Based on a method described by Okhawa et al. and modified by Pulla and Lokesh [40], the extract/fractions were tested for their ability to inhibit $CuSO_4$-induced LDL oxidation by measuring the amount of TBARS formed. Briefly, 0.5 mL of incubated LDL was mixed with 1 mL of KCl (1.15 M) and 2 mL of chilled thiobarbituric acid (TBA) reagent (0.25 M HCl, 15% trichloroacetic acid, 0.38% TBA, and 0.055% butylated hydroxy toluene). The reaction mixtures were kept in a boiling water bath for 60 min at 100°C and absorbance was measured at 570 nm. The amount of TBARS was calculated using a molar extinction coefficient of $1.56 \times 105\ M^{-1}cm^{-1}$ and expressed as nmoles of TBARS/mg LDL protein.

2.17. GC-MS/MS Analysis. Gas chromatography-mass spectrometry (GC-MS) analysis of EAF was performed at the Sophisticated Instrumentation Centre for Applied Research and Testing (SICART), Vallabh Vidyanagar, Anand, Gujarat. Analysis was performed using Autosystem XL with a turbo mass GC-MS spectrometer (Pekin Elmer, USA) coupled with a Quadrupole analyzer. The GC-MS system was equipped with a PE-5MS column packed with 5% phenyl polysiloxane (30 m × 0.25 mm inner diameter). Helium (99.99%) was used as carrier gas at a flow rate of 1 mL/min and a split ratio of 1 : 10. Temperature programming was applied (starting at 78°C for 5 min; and increasing at 10°C/min up to 300°C and held for 20 min). The sample of EAF was prepared by accurately weighing 10 mg in 5 mL of methanol. The solution was filtered through Whatman I filter paper, along with 2 g sodium sulfate to remove the sediments and traces of water in the filtrate and 1 μL of the solution was used for GC-MS analysis. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 20–620 amu. To obtain the spectral data, separated chromatograms of various phytoconstituents were then subjected to mass fragmentation. Mass fragmentation was interpreted by comparing the spectral data with the database of the National Institute of Standards and Technology (NIST) and identifying compounds.

2.18. Statistical Analysis. All tests were performed in triplicates ($n = 3$). Data are expressed as Mean ± SEM. The statistical analysis was performed by one-way ANOVA followed by Dunnett's test using GraphPad Prism version 6.05 for Windows, GraphPad Software, San Diego, CA, USA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Extraction and Fractionation

3.1.1. Preliminary Phytochemical Investigation. Table 1 shows the presence of secondary metabolites in CPRME crude extract and solvent fractions. Qualitative analysis revealed alkaloids, glycosides, triterpenoids, sterols, flavonoids, and phenolic acids depending on the polarity of solvents used for fractionation and the chemical nature of the phytochemicals. The presence of polyphenolic compounds in extract/fractions is also evident from different color bands obtained in the qualitative TLC fingerprinting (Figure 1).

3.2. In Vitro Antioxidant Activity

3.2.1. Total Phenolic and Flavonoid Contents. The TPC and TFC of CPRME and its solvent fractions were measured (Figure 2). The TPC and TFC were calculated using the regression equation of the standard curve plotted using tannic and quercetin as standard. Among the extract/fractions tested, EAF showed the highest amount of TPC (25.7 ± 3.12 mg TAE/g dry fraction), followed by DMF (19.05 ± 3.29 mg TAE/g dry fraction), CPRME (15.38 ± 2.21 mg TAE/g dry extract), and MF fraction (12.0 ± 2.37 mg TAE/g dry fraction). HF fraction showed the lowest amount of TPC (3.73 ± 0.98 mg TAE/g dry fraction) and TFC was not even detected in it. The highest TFC was found in DMF fraction (13.69 ± 1.74 mg QUE/g dry fraction) followed by EAF (11.4 ± 1.88 mg QUE/g dry fraction), CPRME (10.36 ± 1.51 mg QUE/g dry extract), and MF fraction (9.09 ± 0.82 mg QUE/g dry fraction). Aqueous fraction (AF) showed the lowest amount of TPC (7.78 ± 1.43 mg TAE/g dry fraction) and TFC (1.76 ± 0.63 mg QUE/g dry fraction) when compared with their counterparts.

3.2.2. DPPH Radical Scavenging Activity. Figure 3(a) depicts the scavenging potential of CPRME and its solvent fractions in the following order: EAF (ethyl acetate) > DMF (dichloromethane) > CPRME (methanolic crude extract) > MF (methanolic fraction) > AF (aqueous) > HF (n-hexane). IC_{50} of each fraction was calculated using a linear regression equation. The highest DPPH scavenging effect was produced by EAF fraction with an IC_{50} value of 369.87 μg/mL but weaker than ascorbic acid ($IC_{50} = 30.42$ μg/mL), a reference standard (Table 2). Dichloromethane fraction (DMF) also demonstrated a considerable DPPH radical scavenging effect ($IC_{50} = 482.53$ μg/ml). As indicated by IC_{50} values for CPRME, MF, AF, and HF required higher concentrations to scavenge DPPH radicals.

Table 1: Extraction of the yield of CPRME and its solvent fractions.

	CPRME (crude)	Extract/fractions				
		HF	DMF	EAF	MF	AF
Yield (%)	11.75[#]	8.15[*]	2.68[*]	11.05[*]	31.14[*]	23.38[*]
Phytochemicals						
Alkaloids	+	−	+	+	−	−
Carbohydrates	+	−	−	−	+	+
Glycosides	+	−	+	+	+	−
Triterpenes	+	+	+	+	+	−
Sterols	+	+	+	+	−	−
Flavonoids	+	−	+	+	+	+
Phenolics/tannin	+	−	+	+	+	+
Saponins	+	−	−	−	−	+
Proteins	+	−	−	−	−	+

[#]% crude extract yield = wt. of the dry extract/wt. of the dry plant material × 100, [*]% solvent fraction yield = wt. of the dry solvent fraction/wt. of the dry crude extract × 100. CPRME: *Calotropis procera* root bark methanol extract; HF: hexane fraction; DMF: dichloromethane fraction; EAF: ethyl acetate fraction; MF: methanol fraction; AF: aqueous fraction.

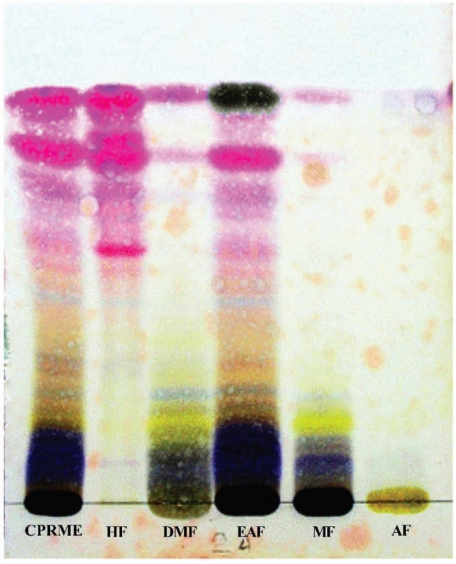


Figure 1: TLC chromatogram of CPRME and its solvent fractions. CPRME: *Calotropis procera* root bark methanol extract; HF: hexane fraction; DMF: dichloromethane fraction; EAF: ethyl acetate fraction; MF: methanol fraction; AF: aqueous fraction.

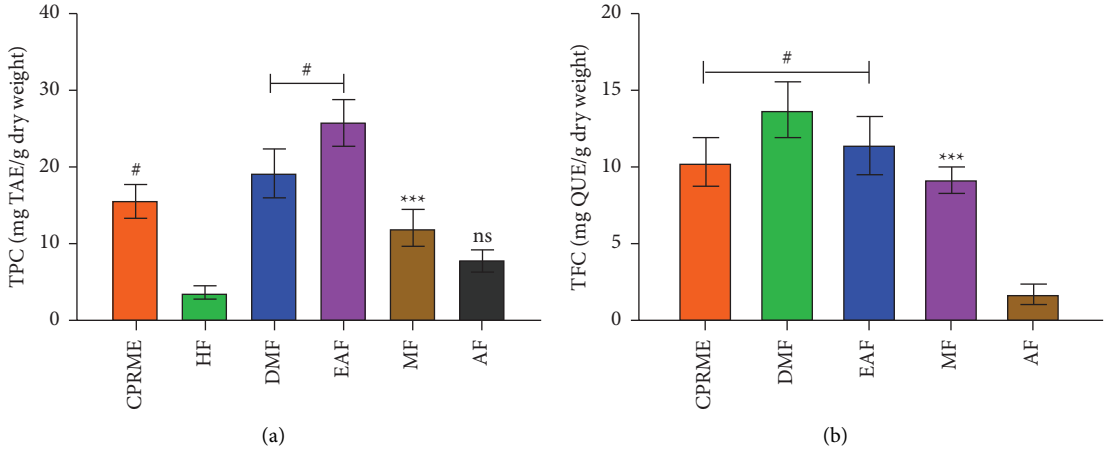


Figure 2: Total phenolic (a) and flavonoid (b) contents of CPRME and its solvent fractions. All tests were performed in triplicates ($n = 3$). For TPC, $^{***}P < 0.001$, $^{\#}P < 0.0001$ when compared with HF. For TFC, $^{***}P < 0.001$, $^{\#}P < 0.0001$ when compared with AF. ns: not significant. TPC: total phenolic content, TFC: total flavonoid content, CPRME: *Calotropis procera* root bark methanol extract; HF: hexane fraction; DMF: dichloromethane fraction; EAF: ethyl acetate fraction; MF: methanol fraction; AF: aqueous fraction.

Table 2: IC_{50} values of CMRME and its solvent fractions on different *in vitro* radical systems tested.

Extract/fractions	DPPH scavenging	NO radical scavenging	H_2O_2 scavenging	$^{\bullet}OH^{-}$ radical scavenging IC_{50} (μg/ml)	$^{\bullet}O_2^{-}$ anion scavenging	Fe^{3+} ion chelating	LDL oxidation inhibitory
CPRME	$619.77 \pm 13.78^{b\#}$	$355.80 \pm 14.30^{b\#}$	$594.06 \pm 11.12^{b\#}$	$635.09 \pm 11.21^{b\#}$	$1029.83 \pm 20.92^{b\#}$	$943.42 \pm 9.44^{b\#}$	$1316.01 \pm 13.14^{b\#}$
HF	2298.29 ± 63.72^{n}	2015.64 ± 43.50^{n}	2245.78 ± 64.67^{n}	4843.65 ± 141.65^{n}	2202.07 ± 55.91^{n}	1374.39 ± 20.62^{n}	$971.54 \pm 9.49^{a\#}$
DMF	$482.53 \pm 12.16^{a*,b\#}$	$249.87 \pm 7.52^{a***,b\#}$	$532.47 \pm 9.32^{ns,b\#}$	$564.81 \pm 10.84^{ns,b\#}$	$657.85 \pm 12.14^{a****,b\#}$	$804.77 \pm 7.91^{a\#,b\#}$	$795.31 \pm 10.21^{a\#,b**}$
EAF	$369.87 \pm 7.58^{a****,b\#}$	$132.31 \pm 5.78^{a\#,b\#}$	$396.85 \pm 6.13^{a***,b\#}$	$195.39 \pm 4.95^{a****,b\#}$	$741.90 \pm 12.5^{a\#,b\#}$	$627.01 \pm 8.99^{a\#,b\#}$	$452.57 \pm 8.61^{a\#,b\#}$
MF	$794.35 \pm 13.43^{n,b\#}$	$542.41 \pm 8.32^{n,b\#}$	$665.14 \pm 8.39^{n,b\#}$	$1196.09 \pm 13.91^{n,b\#}$	$1716.60 \pm 54.34^{n,b\#}$	$614.73 \pm 10.87^{a\#,b\#}$	$1743.17 \pm 22.27^{n,b\#}$
AF	$1568.93 \pm 38.14^{n,b\#}$	$1096.96 \pm 14.95^{n,b\#}$	$887.41 \pm 9.07^{n,b\#}$	$1993.07 \pm 35.20^{n,b\#}$	$1889.88 \pm 47.40^{n,b***}$	$1228.80 \pm 13.07^{n,b\#}$	$2930.62 \pm 62.47^{n,b\#}$
Ascorbic acid (ASA)	$30.42 \pm 2.12^{a\#,b\#}$	$53.81 \pm 3.51^{a\#,b\#}$	$45.38 \pm 2.39^{a\#,b\#}$	$57.23 \pm 3.68^{a\#,b\#}$	$91.53 \pm 4.71^{a\#,b\#}$	$150.03 \pm 3.41^{a\#,b\#}$	NA

IC_{50} values are expressed as mean ± SEM ($n = 3$). IC_{50} of CPRME (crude extract) is compared with that of different solvent fractions. $^{a*}P < 0.05$, $^{a**}P < 0.01$, $^{a***}P < 0.001$, $^{a\#}P < 0.0001$ when compared with CPRME and $^{b**}P < 0.01$, $^{b***}P < 0.001$, $^{b\#}P < 0.0001$ when compared with HF (hexane fraction). ns: not significant. n: indicates a higher IC_{50} than CPRME and therefore, lower radical scavenging activity. NA: no standard was used. The results of LDL oxidation assay of crude extract and solvent fractions were compared with positive control (native LDL with $CuSO_4$ only) CPRME: *Calotropis procera* root bark methanol extract; HF: hexane fraction; DMF: dichloromethane fraction; EAF: ethyl acetate fraction; MF: methanol fraction; AF: aqueous fraction.

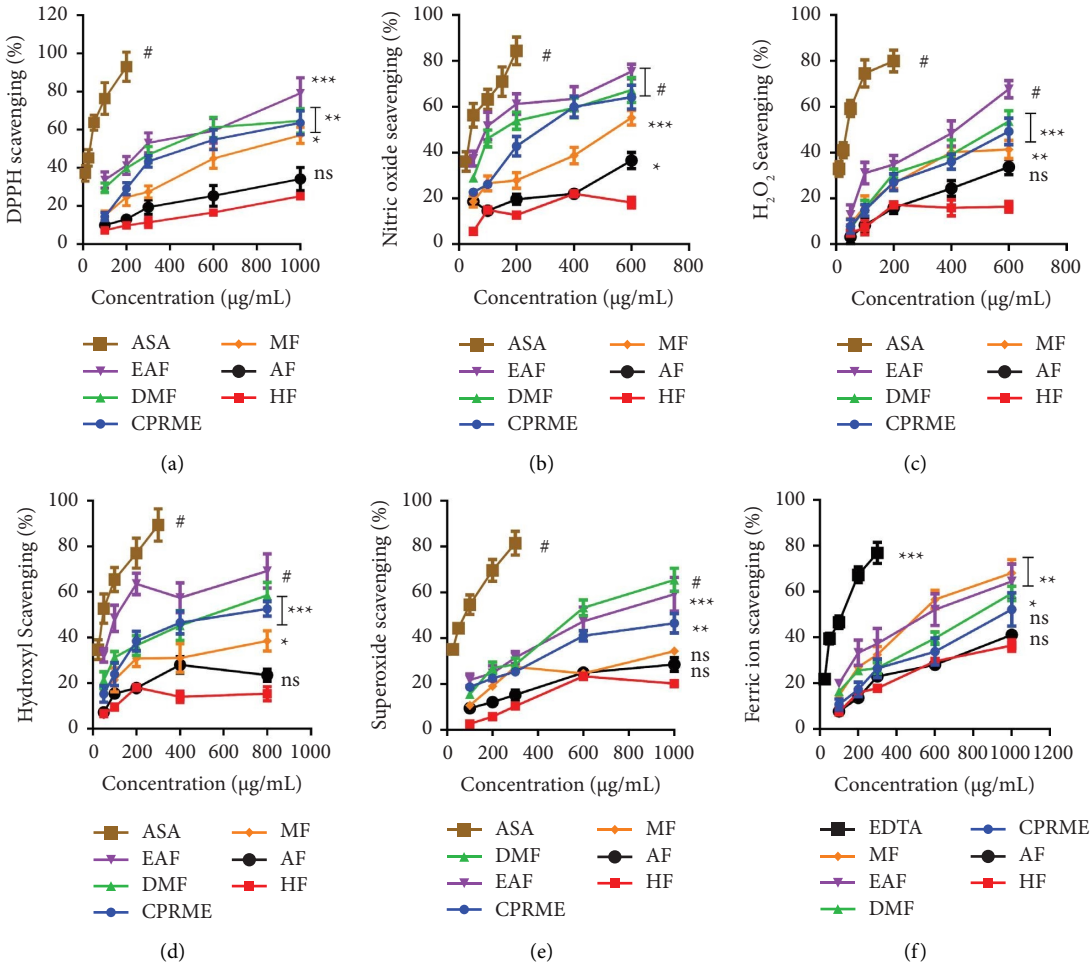


FIGURE 3: (a–f) *In vitro* antioxidant activity of CPRME and its solvent fractions. All tests were performed in triplicates ($n = 3$). The statistical significance of antioxidant activity was analyzed for the highest concentration of extract/fractions/standard used in the respective tests. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.0001$ when compared with HF (the lowest antioxidant activity). ns: not significant. CPRME: *Calotropis procera* root bark methanol extract; HF: hexane fraction; DMF: dichloromethane fraction; EAF: ethyl acetate fraction; MF: methanol fraction; AF: aqueous fraction; ASA: ascorbic acid; EDTA: ethylenediaminetetraacetic acid.

3.2.3. Nitric Oxide Scavenging Activity. The scavenging activity of CPRME and its solvent fractions against nitric oxide released by sodium nitroprusside was investigated by the Griess reagent method (Figure 3(b)). In this study, the NO scavenging capacities of samples and the positive control increased in a dose-dependent manner. The order of efficacy was observed to be EAF > DMF > CPRME > MF > AF > HF in a dose-dependent manner. A % inhibition shown by EAF was highest at the dose of 600 μg/ml (75.39 ± 3.214%) while that of CPRME, DMF, MF, AF, and HF was found to be 64.24 ± 5.16, 67.32 ± 5.44, 55.19 ± 3.22, and 36.57 ± 3.52%, respectively, in decreasing order. These results were further consolidated by IC_{50} analysis data (Table 2), showing the EAF fraction with the highest NO scavenging ability ($IC_{50} = 317.46$ μg/mL) among the fractions.

3.2.4. H_2O_2 Scavenging Activity. Figure 3(c) depicts the % H_2O_2 scavenging ability of CPRME and its solvent fractions. The order of efficacy was: EAF > DMF > CPRME > MF > AF > HF. As evident, EAF required the lowest IC50 concentration ($IC_{50} = 396.85$ μg/mL) followed by DMF ($IC_{50} = 532.47$ μg/mL) and CPRME ($IC_{50} = 594.06$ μg/mL). Other fractions, such as MF ($IC_{50} = 665.14$ μg/mL) and AF ($IC_{50} = 887.41$ μg/mL), required a higher concentration to scavenge H_2O_2, whereas the HF fraction could only provide 50% inhibition at a concentration of 2245.78 μg/mL (Table 2). Except for HF, all fractions displayed reasonable H_2O_2 scavenging activity, though lower than the reference drug ascorbic acid ($IC_{50} = 57.23$ μg/mL).

3.2.5. Hydroxyl Radical Scavenging Activity. Figure 3(d) shows the efficacy of CPRME and its solvent fractions in scavenging hydroxyl radicals in the order of EAF > DMF > CPRME > MF > AF > HF. Among the fractions, EAF was found to be more powerful as a hydroxyl radical scavenger followed by DMF and CPRME. The IC_{50} values (Table 2) ranged from 195.39 μg/mL (for EAF) to 4843.65 μg/mL (for HF). The MF, AF, and HF needed considerably higher concentrations to scavenge 50% hydroxyl radicals. The hydroxyl radical scavenging potency of

EAF was the highest among the fraction but lower than the reference drug ascorbic acid ($IC_{50} = 57.23\,\mu g/mL$).

3.2.6. Superoxide Radical Scavenging Activity. Figure 3(e) highlights the trends of CPRME and its solvent fractions in quenching superoxide anion. Their potency as a superoxide radical scavenger was in the order of DMF > EAF > CPRME > MF > AF > HF. From the analysis of IC_{50} values (Table 2), the SO radical scavenging potential of DMF ($IC_{50} = 657.85\,\mu g/mL$) was found to be higher followed by EAF ($IC_{50} = 741.90\,\mu g/mL$) but less when compared with positive control ascorbic acid ($IC_{50} = 91.53\,\mu g/mL$). CPRME, MF, AF, and HF required greater than $1000\,\mu g/mL$ concentration to neutralize superoxide radicals.

3.2.7. Metal Chelating Activity. The ferrous ion chelating ability of various solvent fractions of CPRME was studied using the ferrozine-Fe^{2+} complex method (Figure 3(f)). The order of efficacy was MF > EAF > DMF > CPRME > AF > HF. MF, EAF, DMF, and CPRME showed the highest iron chelating potency with IC_{50} of 614.73 $\mu g/mL$, 627.01 $\mu g/ml$, 804.77 $\mu g/mL$, and 943.42 $\mu g/mL$. Other fractions, AF and HF, showed iron chelation at slightly higher than $1000\,\mu g/mL$ concentration (Table 2). EDTA, a reference standard, showed the lowest IC_{50} values ($150.03\,\mu g/mL$).

3.3. Total Antioxidant Capacity. Figure 4 shows the total antioxidant capacity (TAC) of solvent fractions of CPRME. The total antioxidant activity is expressed as mg ascorbic acid equivalents (AAE). TAC was in the order of EAF > DMF > CPRME > MF > AF > HF. The TAC for EAF was found to be 94.14 ± 9.11 mg AAE, the highest among the fractions. This was followed by DMF (68.10 ± 8.78 mg AAE), CPRME (57.21 ± 5.66 mg AAE), MF (41.80 ± 4.69 mg AAE), AF (32.07 ± 7.87 mg AAE) and HF (16.06 ± 1.13 mg AAE).

3.4. Reducing Power Assay. Figure 5 depicts the reductive power of CPRME and its different solvent fractions. An increase in absorbance is suggestive of higher reducing power. The results showed a concentration-dependent increase in the absorbance at 700 nm for extract fractions and the positive control ascorbic acid. Ascorbic acid showed a very powerful reducing ability. EAF containing a good amount of TPC and TFC was more powerful in reducing Fe^{3+} to Fe^{2}, followed by DMF, CPRME crude extract, MF, and AF. A negligible reducing activity was observed with HF.

3.5. LDL Protective Effects. Since oxidized LDL (ox-LDL) has been implicated in the initiation and progression of atherosclerosis, hyperlipidemic agents with additional LDL protective effects would be more appreciated than the agents with only hyperlipidemic effects. For this reason, we also measured the protective effect of CPRME and its different solvent fractions on copper-induced LDL oxidation in the present study. Different concentrations of CPRME and its solvent fractions (25, 50, 100, 200, 500, 1000, and 1500 $\mu g/ml$) were pre-incubated with LDL

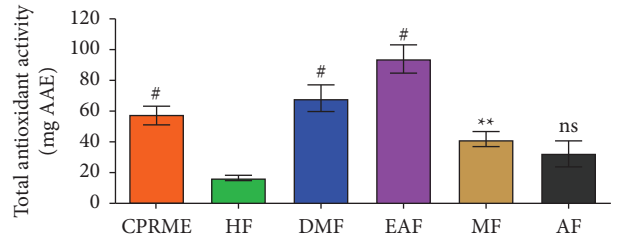


Figure 4: Total antioxidant activity of CPRME and its solvent fractions. All tests were performed in triplicates ($n = 3$). $^{**}P < 0.01$, $^{\#}P < 0.0001$ when compared with HF (the lowest total antioxidant activity). ns: not significant. CPRME: *Calotropis procera* root bark methanol extract; HF: hexane fraction; DMF: dichloromethane fraction; EAF: ethyl acetate fraction; MF: methanol fraction; AF: aqueous fraction.

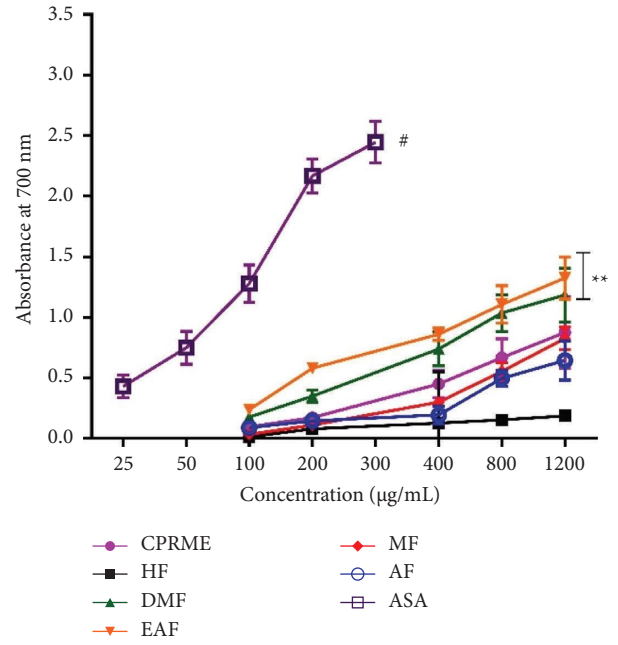


Figure 5: Reducing power of CPRME and its solvent fractions. All tests were performed in triplicates ($n = 3$). The statistical analysis of reducing power at the highest concentration of extract/fractions ($1200\,\mu g/mL$) and standard ($300\,\mu g/mL$) was performed. $^{**}P < 0.01$, $^{\#}P < 0.0001$ when compared with HF (the lowest reducing power). CPRME: *Calotropis procera* root bark methanol extract; HF: hexane fraction; DMF: dichloromethane fraction; EAF: ethyl acetate fraction; MF: methanol fraction; AF: aqueous fraction.

followed by $CuSO_4$ modification for 6 h (Figure 6). In this study, we have kept two additional groups: negative control (only native LDL without $CuSO_4$ and sample treatment) and positive control (native LDL with $CuSO_4$ only). Post 6 h of incubation of LDL with $CuSO_4$, there was a significant increase ($P < 0.001$) of thiobarbituric acid reactive substances (TBARS) in the reaction mixture, suggesting copper-induced oxidative modification LDL when compared with normal control tubes. The inhibitory efficacy of the CPRME (crude) and fractions on LDL modification is in the following order: EAF > DMF > HF > CPRME > MF > AF ($P < 0.01$ for AF, $P < 0.001$ for all other groups) when compared with positive control. EAF significantly

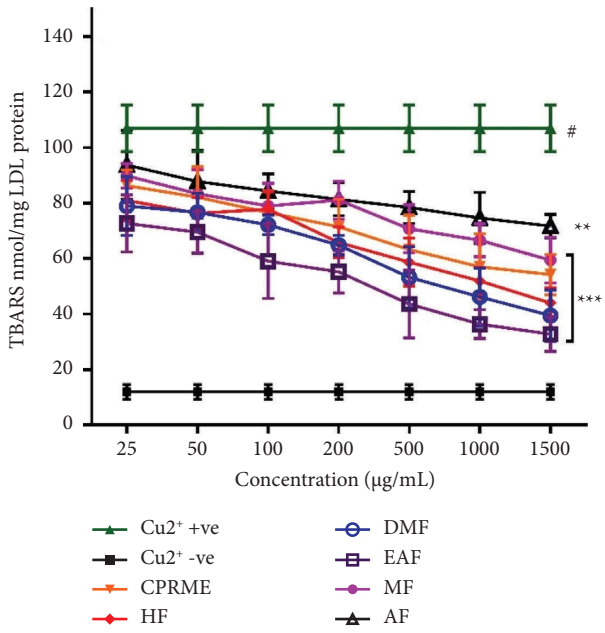


Figure 6: *In vitro* LDL protective effect of CPRME and its solvent fractions. CPRME: *Calotropis procera* root bark methanol extract; HF: hexane fraction; DMF: dichloromethane fraction; EAF: ethyl acetate fraction; MF: methanol fraction; AF: aqueous fraction. All tests were performed in triplicates ($n = 3$). Data were expressed as mean ± SEM. Statistical analysis of TBARS value at the highest concentration of extract/fractions (1500 μg/mL) was performed. $^{\#}P < 0.001$ when positive control (native LDL with $CuSO_4$ only) compared with negative control (native LDL without $CuSO_4$ and sample treated), $^{**}P < 0.01$, $^{***}P < 0.001$ when test extract/fractions compared with positive control (native LDL with $CuSO_4$ only).

reduced the formation of TBARS in a dose-dependent manner when compared with positive control while DMF, HF, and CPRME required higher concentrations to produce the same effect observed with EAF.

3.6. GC-MS/MS Analysis of EAF. Figure 7 shows the gas chromatogram of EAF. The gas chromatography-mass spectrometry (GC-MS) analysis of EAF revealed the presence of important secondary metabolites which were identified by comparing their mass spectral fragmentation patterns with those of known compounds listed in the National Institute of Standards and Technology (NIST) library. Table 3 highlights major phytochemicals identified are lup-20(29)-en-3-ol acetate (43.71), 3-O-acetyl-6-methoxycycloartenol (19.98), β-amyrin (14.21), α-amyrin (7.30), betulin (3.52), and urs-12-en-28-oic acid (2.43). The mass fragmentation spectra (Figures S1–S6) have been provided as supplementary material.

4. Discussion

Antioxidants contribute to the removal of these oxidative products and slow down the process of oxidative modification and thereby preventing damage to the biological macromolecules such as cellular proteins, lipids and nucleic acids, and disruption of mitochondrial respiration [41]. Polyphenols found in plants are secondary metabolites that have one or more hydroxyl groups attached to one or more aromatic rings. Several researchers have demonstrated that these plant polyphenols can serve as antioxidants to combat oxidative stress-induced diseases [42]. In addition to polyphenolics, pentacyclic triterpenes, a group of secondary plant metabolites such as lupeol, α-amyrin, β-amyrin, and ursolic acid are also reported to have antioxidant properties and are valuable as antiinflammatory, antihypertensive, antiviral, antiangiogenic, antitumor, and antiangiogenic agents [24]. These antioxidant compounds can scavenge radical oxygen species such as superoxide, hydroxyl, nitric oxide, and peroxides and consequently impede pathogenic oxidative processes of chronic diseases such as cardiovascular diseases, diabetes, neurodegenerative diseases, and cancer [1]. Herbs have been regarded as effective antioxidants since ancient times. In the present study, total phenolic and flavonoid contents were estimated in crude extract/fractions. The results indicate that polyphenolic compounds were accumulated in nonpolar and semipolar fractions. EAF showed the highest phenolic content while flavonoid content was higher in DMF. The results are in agreement with the previously reported studies [43, 44].

For the evaluation of the free radical scavenging abilities of bioactive fractions of plant extracts and foods, the DPPH (1,1-diphenyl 1-2-picryl-hydrazyl) test has been widely utilized in phytomedicine. DPPH which is a stable nitrogen-centered radical can accept hydrogen atoms from antioxidants to form a stable diamagnetic molecule [45]. Therefore, when an antioxidant reacts with DPPH, the electron is paired off and the DPPH solution decolorizes. This reaction yields a stable product 1,1-diphenyl-2-picryl hydrazine by donating hydrogen atoms, resulting in a color change from purple to yellow which can be monitored as a decrease in absorbance at 517 nm [46–48]. The number of electrons taken up determines the antioxidant's ability to scavenge free radicals or bleach the color stoichiometrically [45]. In the present study, the CPRME and subfractions were found to have varied amounts of phenolic and flavonoid contents. The results suggest that solvent fractions EAF and DMF followed by CPRME crude extract showed significantly higher $DPPH^+$ scavenging activity that positively correlated with their phenolic compounds such as flavonoids, tannins, and phenolic acids. The EAF contained the highest total phenolic content followed by DMF, whereas total flavonoid content was higher in DMF than in EAF. These results are in accordance with the previously reported studies [43, 44]. The hydroxyl groups (-OH) present in these polyphenolic compounds have the ability to donate hydrogen atoms to the DPPH radical, thereby neutralizing it. The antioxidant activity of these compounds depends on the molecular structure, in particular on the number and position of hydroxyl groups and the nature of substitutions on the aromatic rings [49]. Some flavonoids such as rutin, isorhamnetin, and dihydroquercetin have been reported from *C. procera* root bark [50, 51] and have efficient antioxidant activity [52–54]. Therefore, the observed $DPPH^+$

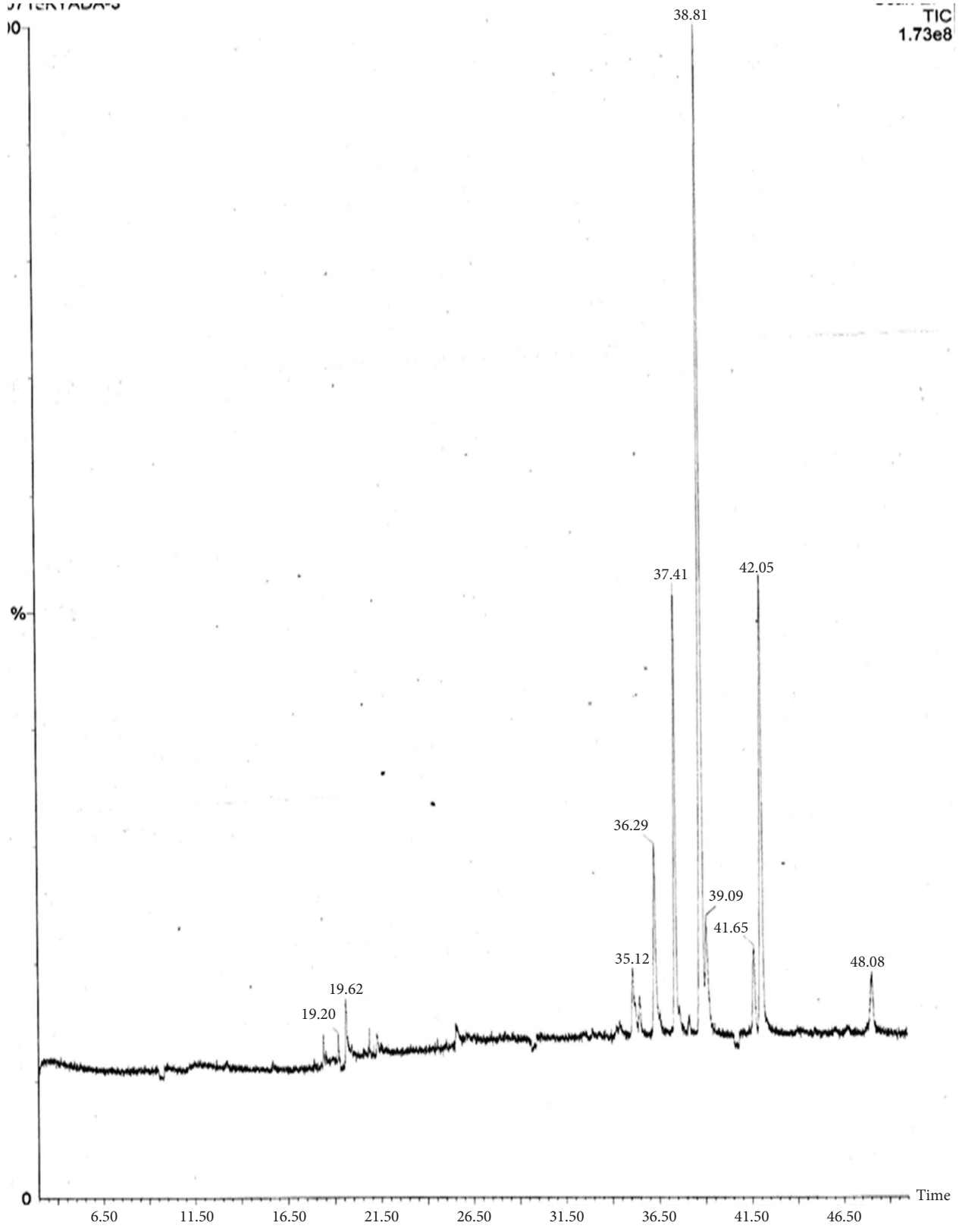
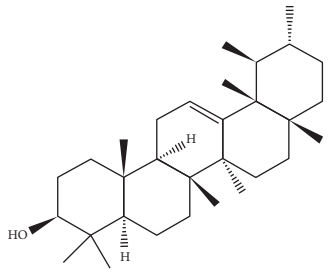
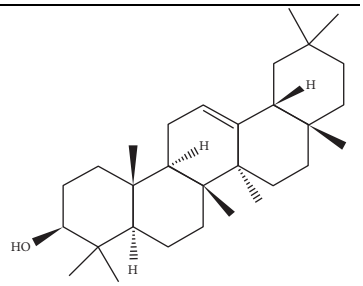
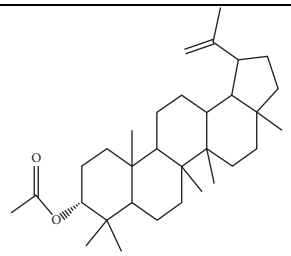
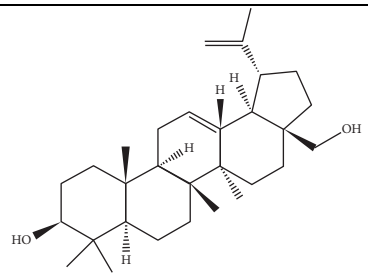
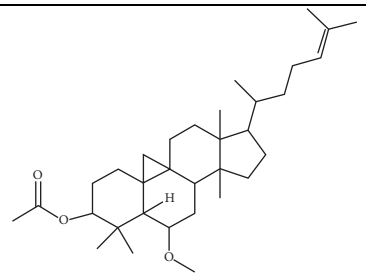
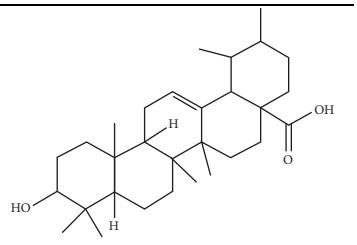


Figure 7: GC-MS chromatogram of EAF.

scavenging activity may probably be due to the hydrogen-donating capability of the polyphenolic compounds present in EAF and DMF [55].

Nitric oxide (NO) is an essential bifaceted bioregulatory molecule with many physiological functions and pathological implications. Physiological effects include smooth

Table 3: GC-MS analysis of EAF depicting retention time (RT), peak area, and compounds identified.

Peak no.	RT	Peak area	Height	Compounds	Area %	Structure of the compound
6	36.29	3404640.8	25359984	α-amyrin	7.30	
7	37.41	6628454.5	62165052	β-amyrin	14.21	
8	38.81	20388076.0	140033936	Lup-20(29)-en-3-ol acetate	43.71	
10	41.65	1640717.8	11566819	Betulin	3.52	
11	42.05	9317410.0	64812176	3-O-acetyl-6-methoxy-cycloartenol	19.98	
12	48.08	1133531.9	7151552	Urs-12-en-28-oic acid	2.43	

muscle relaxation, blood pressure regulation, inhibition of platelet aggregation, neural signal transmission, and immune response [56]. It has a very short half-life and may act as a highly unstable NO radical. Although NO does not interact with biological macromolecules directly, excessive superoxide anions in some chronic diseases react with NO to form peroxynitrite anion ($^{\bullet}ONOO^{-}$), which prevent sodium transport across membranes, inhibit mitochondrial respiratory chain enzymes, and reduce cellular oxygen consumption. These anions even cause neuronal damage and DNA fragmentation and also participate in the pathogenesis of inflammation [57, 58]. The nitric oxide radical scavenging activity of CPRME crude extract and solvent fractions was measured by the Greiss method. $NO^{\bullet}$, spontaneously generated in the aqueous solution of sodium nitroprusside at physiological pH, could interact with oxygen to produce nitrite ions, causing diazotization of sulphanilamide. The diazotized product undergoes coupling with naphthylethylenediamine dichloride forming an azo-dye, the intensity of which is measured at 550 nm. Phytochemicals with nitric oxide radical scavenging activity would compete with oxygen for nitric oxide thereby inhibiting the production of nitrite ions. This leads to a decrease in absorbance at 550 nm [58]. In the present study, solvent fractions EAF and DMF showed considerable $NO^{\bullet}$ scavenging effect followed by CPRME crude extract. Polyphenols are known to have antioxidant and free radical scavenging efficacy1. Due to the presence of aromatic structural elements, multiple hydroxyl groups, and a highly conjugated system, they can effectively scavenge reactive oxygen species (ROS). These free radicals can be neutralized by polyphenols by generating stable chemical complexes that halt subsequent detrimental processes. [59]. The free radicals nitric oxide and singlet oxygen (O–) participate in lipid peroxidation and are also implicated in the pathogenesis of chronic inflammation [60]. Polyphenols can inhibit the activity of inducible nitric oxide synthase (iNOs) or act as free radical scavengers to mitigate the propagation of inflammation by NO [59]. The $NO^{\bullet}$ scavenging property of EAF and DMF could be valuable in iNOS-generated excessive $NO^{\bullet}$ radicals during chronic inflammation in conditions such as atherosclerosis.

H_2O_2 is a slowly oxidizing, non-free radical species, but a substantial source of damaging hydroxyl radicals ($OH^{\bullet}$). It can directly inactivate some enzymes by oxidation of essential thiol (-SH) groups. H_2O_2 can cross cell membranes rapidly and participate in Fe^{2+} and Cu^{2+}-catalyzed Fenton reactions, giving rise to destructive hydroxyl radicals within the cells. The resultant hydroxyl radicals may react with most of the biomolecules causing cell injury [61, 62]. It is, therefore, biologically advantageous for cells to regulate the amount of H_2O_2 by decomposing it into oxygen and water. The decomposition of H_2O_2 by EAF and DMF was highest in comparison to other fractions.

Hydroxyl radical is regarded as the most reactive oxidant among ROS, causing lipid peroxidation to produce lipid hydroperoxides by abstracting hydrogen atoms from membrane polyunsaturated fatty acid and causing enormous biological damage in living systems [63, 64]. Lipid hydroperoxides are later decomposed to alkoxyl and peroxyl radicals and maintain the vicious cycle of oxidative cellular damage [65, 66]. In addition, it is a potent cytotoxic agent, capable of attacking the majority classes of biomacromolecules including proteins, DNA, and lipids and contributes to the pathological progression of many diseases such as atherosclerosis, diabetes, ischemic heart disease, cancer, Alzheimer's disease, and aging [67, 68]. Thus, it is very crucial to keep the hydroxyl radical at a minimum level for the protection of biological systems. Phenolic compounds with multiple hydroxyl groups exhibit high redox potential. The antioxidant properties of phenolic compounds are significantly influenced by the substituents on the phenyl ring and the conjugated carbon skeleton. [69]. Due to the protective functions that antioxidants play in biological systems, investigating the radical scavenging and reducing capacities of antioxidants, especially those that are naturally present in plant sources is of significant interest. The most common reaction mechanism proposed for scavenging hydroxyl (OH) radicals may include hydrogen atom transfer (HAT) by natural polyphenolic compounds. It has been reported that the most active site of $^{\bullet}OH$ scavenging by polyphenols is the –OH group in the benzene ring by hydrogen atom transfer HAT mechanism [70]. The capability of solvent fractions EAF and DMF followed by CPRME crude extract to eliminate hydroxyl radicals could provide significant protection to biomolecules and impair pathobiological mechanisms implicated in chronic disorders.

Superoxide radicals although less reactive than hydroxyl radicals, which are continually produced during normal mitochondrial oxidative metabolism. They serve as precursors to the majority of ROS species and mediate oxidative chain events [71, 72]. However, excessively generated superoxide radicals are known to be damaging to biomolecules directly or indirectly by forming harmful H_2O_2, $OH^{\bullet}$, peroxynitrite anion ($^{\bullet}ONOO^{-}$), or singlet oxygen during pathological events such as ischemic reperfusion injury [73]. It was assumed that $^{\bullet}O_{2-}$ could be the primary target of antioxidants against oxidative stress, indirectly decreasing the levels of other reactive species in the biological systems [71]. Reduction of flavins in the presence of light generates superoxide radicals which reduce nitroblue tetrazolium (NBT, yellow color), to blue-colored formazan which can be measured at 570 nm [33]. The present study suggests that DMF has the highest superoxide radical scavenging effect followed by EAF due to the presence of polyphenolic compounds; however, higher concentrations are required to quench the $^{\bullet}O_{2-}$ radicals. It is suggested that plant polyphenols can mimic SOD enzyme action through the π-π interaction between superoxide and one polyphenol ring and is linked to oxidation of the superoxide radical, due to the transfer of its unpaired electron to an aromatic ring of the polyphenol and subsequent O_2 release during the reaction [74].

Through the Fenton reaction, the ferrous ion (Fe^{2+}) can initiate lipid peroxidation by the breakdown of H_2O_2 and decomposing lipid hydroperoxides into reactive hydroxyl-, peroxyl-, and alkoxyl-free radicals. This reaction gives

momentum to the chain reaction of lipid peroxidation by abstracting hydrogen atoms from other vital molecules [75, 76]. The metal chelating capacity of a compound eliminates excess catalyzing transition metals which is critical in the prevention of lipid peroxidation. It is well established that chelating agents decrease the redox potential by forming disulfide bonds with metal, thereby rendering the oxidized metal ion into a nonreactive form [77]. Ferrozine can make a complex with ferrous ions producing a purple color product. In the presence of chelating agents/extracts, the intensity of the purple color complex is reduced in a dose-dependent manner. This suggests that the plant components may either interfere in ferrozine-Fe^{2+} complex formation or directly interact with Fe^{2+}. Thus, the chelating effect of plant extracts as natural chelators can be determined by measuring the intensity of the purple color formed [78]. A decrease in color intensity quantitatively correlates with higher metal chelating ability. Chelating agents that form bonds with metal are effective in reducing the redox potential and thereby stabilizing the oxidized form of the metal ions [79]. Polyphenols are very effective metal chelators. Polyphenol-iron interactions (binding) have been proposed as a mechanism for the antioxidant behavior of the polyphenols. In a process known as autooxidation, polyphenol ligand complexes of Fe^{2+} rapidly oxidize in the presence of oxygen to yield Fe^{3+}-polyphenol complexes. The binding of polyphenol ligands to Fe^{2+} lowers the reduction potential of iron and accelerates the rate of iron oxidation. The higher stability of the harder Fe^{3+} metal ion interactions with the hard oxygen ligands of the polyphenol moieties, as well as the strong electron-donating properties of the oxygen ligands that stabilize the higher iron oxidation state, both contribute to this oxidation of Fe^{2+} to Fe^{3+} upon binding to polyphenol ligands [80]. In this study, solvent fractions, notably EAF and MF have shown reasonable metal chelating activity, which may partly be due to interference of phytochemicals with Ferrozine-Fe^{2+} complex formation, thereby establishing their role in the metal chelating activity.

The phosphomolybdenum assay was used to assess the total antioxidant capacity of the crude extracts and fractions. This assay is based on the reduction of phosphomolybdanum ion from Mo (VI) to Mo (V) in the presence of an antioxidant with subsequent formation of a green phosphate/MoV complex with maximal absorption at 695 nm under acidic conditions [81, 82]. In the present study, The TAC for EAF was the highest among the fractions followed by DMF and CPRME (crude extract). The observed effect could be attributed to their vast array of polyphenols and antioxidant triterpene compounds.

The reducing power assay serves as a significant indicator of the overall antioxidant potential of plant extracts. Redox property, hydrogen donating ability, chain breaking potential during free radical generation, quenching transition-metal ions, decomposition of hydroperoxides, inhibition of hydrogen extractions from biomolecules, radical scavenging, and reductive capacity have been proposed mechanisms contributing to the overall antioxidant potential of a plant [83, 84]. The presence of various phenolic compounds, such as phenolic acids, flavonoids, and tannins is typically linked to the reducing capabilities. They have a remarkable free radical chain-breaking ability by donating a hydrogen atom or reacting with certain precursors of peroxide, thus preventing peroxide formation [85, 86]. The presence of reducing agents in plant extracts causes a reduction of the Fe^{3+}-ferricyanide complex to the ferrous form leading to a color change from yellow to various shades of green and blue (Perl's Prussian blue) depending upon the number of reductants present. The concentration of Fe^{2+} in the system is directly proportional to the absorbance of the blue-green solution measured at 700 nm. Therefore, an increased absorbance is indicative of higher reducing power and the ability of a compound to donate electrons is suggestive of its antioxidant potency [87]. In the present investigation, EAF containing a good amount of TPC and TFC were more powerful in reducing Fe^{3+} to Fe^{2+}, followed by DMF and CPRME (crude extract).

Lipid peroxidation is the ultimate result of oxidative damage to polyunsaturated fatty acids transforming membrane lipids into lipid peroxide radicals. Peroxidation of membrane lipids may disrupt membrane transport proteins, change the stability of ligand-binding sites on the membrane, and deactivate membrane-associated enzymes consequently, leading to cell death [88]. In the present study, we also studied the effect of CPRME and solvent fractions on the copper-catalyzed human LDL oxidation, as assessed by the formation of a thiobarbituric acid reactive substance (TBARS). We found that CPRME crude extract, EAF, and DMF significantly counteracted the formation of TBARS. It is believed that the copper ion interacts with lipoprotein and degrades lipid hydroperoxides through a Haber–Weiss type reaction pathway. This phenomenon results in the formation of $OH^{\bullet}$, $RO^{\bullet}$, and $ROO^{\bullet}$ radicals by abstracting a hydrogen atom from a fatty acid side chain at, or near the lipoprotein surface [89–91]. The results obtained from the present study indicate that CPRME and their solvent fractions are capable of affecting the rate of LDL oxidation at the end of 6 hr incubation. This could be due to the ability of the extracts to chelate or interfere with copper, thereby inactivating the redox mechanism and free radical generation in the assay system [92]. We observed that the ethyl acetate fraction showed maximum inhibition of LDL oxidation. This could probably be due to the presence of moderately nonpolar components of EAF which may specifically interact with some lipogenic radicals generated during copper-catalyzed oxidative processes within the hydrophobic core of LDL [93]. Therefore, in the present investigation, the LDL protective effect of CPRME (crude) and its solvent fractions, specifically EAF, DMF, and HF could also be attributed to the stabilization of the outer layer of LDL phospholipids. According to Brown et al., lipophilic phenolic compounds can be localized to the surface of phospholipid bilayers, which can protect against free radical attacks and perhaps sequester metal ions [94]. On the other hand, hydrophilic antioxidants could not optimally access the lipid moiety of LDL and would thus be less effective in countering lipophilic radicals. These arguments on solubility and partitioning behavior of antioxidants suitably support the observed LDL

protective effect produced by EAF, DMF, and HF owing to their polar and nonpolar phytoconstituents having radical scavenging and transitional metal ion chelating actions. This effect could be useful in delaying atheromatous plaque development.

For the chromatographic separation, identification, and quantification of phytoconstituents, gas chromatography with mass spectrometry is known to be an excellent and widely used technology. In this study, EAF showed significant *in vitro* antioxidant activity. GC-MS/MS analysis of EAF led to the identification of therapeutically important phytochemicals. Major phytosterols identified are lup-20(29)-en-3-ol acetate (43.71), cyclolanost-24-en-3-ol acetate (19.98), β-amyrin (14.21), α-amyrin (7.30), betulin (3.52), and urs-12-en-28-oic acid (2.43). Lupeol and its esters exhibited antioxidant [95, 96], hypotensive [97, 98], antihyperglycemic [99, 100], antidyslipidemic [100], potent anti-inflammatory activity [101, 102], antiangiogenic [103], anticancer [104], and nephroprotective [105] activities. Lupeol and lupeol linoleate are also demostrated protective role against hepatic lipemic-oxidative injury and lipoprotein peroxidation in experimental hypercholesterolemia [106]. Cycloartenol and its esters exhibited antibacterial, antioxidant, wound healing, topical anti-inflammatory [107], and antimalarial activity [108]. α- and β-amyrin are reported to have antioxidant activities [109, 110]. The therapeutic potential of α- and β-amyrin have been confirmed by previous studies demonstrating analgesic, anti-inflammatory, anticonvulsant, antidepressant, gastro-protective, hepatoprotective, antipancreatitis [111], anti-hyperglycemic, and hypolipidemic effects [112]. Ursolic acid is documented for its important biological effects, including antioxidant, anti-inflammatory, antidiabetic, antibacterial [113], antioxidant [114], and anticancer [115, 116] activities.

5. Conclusion

The antioxidant potential of different solvent fractions of *C. procera* root bark methanol extract was evaluated using different radical systems. In this radical scavenging mechanism, polyphenols sacrificially reduce ROS/RNS, such as $^{\bullet}OH$, $^{\bullet}O_2^-$, $NO^{\bullet}$, or OONO− after generation, preventing damage to biomolecules, or formation of more reactive ROS. The present study confirms the free radical scavenging activity of crude extract and solvent fractions of C. *procera* root bark, notably EAF and DMF. The observed antioxidant properties may be attributable to various secondary metabolites as these fractions contain high polyphenolic and pentacyclic triterpene compounds which correlate with their good antioxidant efficacy. The presence of antioxidant pentacyclic triterpenes such as lupeol, α-amyrin, β-amyrin, and ursolic acid in EAF was characterized using GC-MS/MS approach. The total phenolic content was highest in EAF followed by DMF whereas total flavonoid content was highest in DMF. The EAF produced significant $DPPH^+$, $NO^{\bullet}$, H_2O_2, and $OH^{\bullet}$ scavenging activity. Apart from that, DMF was more effective in $^{\bullet}O_2^-$ scavenging activity while the MF showed the highest metal chelating activity. However, the EAF showed the highest TAC, probably due to the presence of antioxidants pentacyclic triterpenes such as lupeol and amyrins in addition to the polyphenolic compounds. *In vitro* antioxidant studies are relevant in exploring the potential health benefits of plant extracts or their solvent fractions. There is a growing interest within the community in using plant-based antioxidant therapies as complementary and alternative treatments, even in chronic diseases. The current study demonstrates that the phenolic acids, flavonoids, and pentacyclic triterpenes of EAF of *C. Procera* root bark methanol extract can afford the protection against free radicals-induced damage in many chronic health conditions and could be valuable in the prevention and treatment of many chronic diseases such as hyperlipidemia and atherosclerosis. Further studies are required to explore its therapeutic potential.

Data Availability

The data used to support the study are included in the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

A.K. and M.R2. conceptualized the study; D.S., N.V., M.R6., and L.B. proposed the methodology; M.M.A. and S.A.F. managed the software; S.A.F., M.R2., and I.P. validated the study; M.M.A. and S.A.F. formally analyzed the study; A.K., D.S., N.V., M.R6., and L.B. investigated the study; S.A.F and M.M.A. gathered the resources; S.A.F. and M.R2. curated the data; A.K. wrote the original draft; S.A.M., M.R2., and I.P. wrote the review and edited the manuscript; M.R2. and I.P. visualized the study; A.K. and M.R2. supervised the study; A.K. and M.M.A. administered the project; M.R2 acquired the funding. All authors have read and agreed to the published version of the manuscript.

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Supplementary Materials

The following supporting information can be downloaded. Figure S1: mass fragmentation pattern of peak 6 of the gas chromatogram, Figure S2: mass fragmentation pattern of peak 7 of the gas chromatogram, Figure S3: mass fragmentation pattern of peak 8 of the gas chromatogram, Figure S4: mass fragmentation pattern of peak 10 of the gas chromatogram, Figure S5: mass fragmentation pattern of peak 11 of the gas chromatogram, and Figure S6: mass fragmentation pattern of peak 12 of the gas chromatogram. (*Supplementary Materials*)

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