Article

Unraveling Compositional Study, Chemometric Analysis, and Cell-Based Antioxidant Potential of Selective High Nutraceutical Value Amaranth Cultivars Using a GC-MS and NMR-Based Metabolomics Approach

Annie Gupta,* Gaurav Deep Singh, Arti Gautam, Tusha Tripathi, Amit Kumar Taneja, Brahma N Singh, Raja Roy, Om P. Sidhu, Sunil Kumar Panda,* and Arun Bhatt
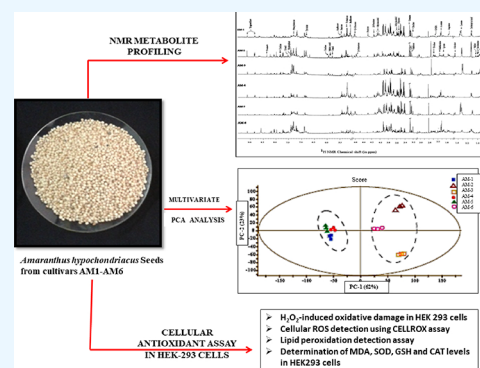
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ABSTRACT: *Amaranthus* (family Amaranthaceae) is a potentially nutritious pseudocereal also known as a functional food owing to its high nutritional quality grains especially rich in essential amino acids. Emerging study, however, unambiguously indicates that apart from essential nutrients like protein, other phytochemicals present in amaranth seeds provide excellent health benefits. Squalene is one such phytonutrient found in *Amaranthus* seeds, which is also its largest vegetal source. In this research work, GC-MS and NMR spectroscopy-based metabolomics have been utilized for the compositional analysis of *Amaranthus* seeds coupled with a multivariate data set. Investigation of nonpolar and polar seed extracts of six different cultivars of amaranth identified 47 primary and secondary metabolites. One-way ANOVA showed significant quantitative metabolic variations in different cultivars of amaranth. Multivariate principal component analysis of both the GC-MS and NMR analyzed data broadly classified in two groups showed significant variations in the polar (lysine, arginine, GABA, and myoinositol) and nonpolar (squalene, tryptophan, and alkylated phenols, which are potential nutraceutical agents) metabolites. The squalene content estimated using HPLC varied significantly (1.61 to 4.72 mg g^{-1} seed dry weight) among six different cultivars. Positive correlations were found among the cellular antioxidant activity and squalene content. Cultivar AM-3 having the maximum squalene content showed the highest antioxidant activity evaluated on the cellular level over human embryonic kidney cells, clearly revealing potent intercellular reactive oxygen species scavenging capacity and strong membrane lipid peroxidation inhibition potential. Oxidative stress markers such as MDA, SOD, GSH, and CAT levels in cells further corroborated the research work. The study also indicated high concentrations of lysine (80.49 mg g^{-1} dry seeds) in AM-2, squalene (0.47% by weight) in AM-3, and 2,4-di-*tert*-butyl phenol (18.64% peak area) and myoinositol (79.07 mg g^{-1} dry seeds) in AM-5. This novel comparative metabolomic study successfully profiles the nutrient composition of amaranth cultivars and provides the opportunity for the development of nutraceuticals and natural antioxidants from this functional food.

Amaranthus hypochondriacus Seeds from cultivars AM1-AM6

NMR METABOLITE PROFILING

MULTIVARIATE PCA ANALYSIS

CELLULAR ANTIOXIDANT ASSAY IN HEK-293 CELLS
➢ H$_2$O$_2$-induced oxidative damage in HEK 293 cells
➢ Cellular ROS detection using CELLROX assay
➢ Lipid peroxidation detection assay
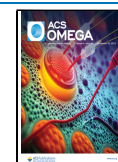➢ Determination of MDA, SOD, GSH, and CAT levels in HEK293 cells

1. INTRODUCTION

Recent scientific advances in human health and nutraceutical research studies in food sciences have elevated the status of food from simple energy sources to potent and safe alternatives in maintaining health and ameliorating disease risks. An entire food group, namely, functional food, has succeeded in achieving a significant place in human diet worldwide owing to its high nutritional and therapeutic qualities[1]. *Amaranthus* seeds (Amaranthaceae) are one such superfood that has attracted increasing attention of researchers recently owing to their gluten free merits and unmatched nutritious functional quality as compared to other cereals. *Amaranthus* seeds are a rich vegetable source of a plethora of phytonutrients such as

unsaturated hydrocarbon squalene, protein, dietary fiber, saturated and unsaturated fatty acids, high vitamins such as riboflavin, niacin, and ascorbic acid, and minerals, especially calcium and sodium. It also have a balanced amino acid content with higher amounts of essential amino acids such as lysine than conventional cereals.[2] Squalene is a poly-

unsaturated triterpene hydrocarbon known for its excellent antioxidant, lipid-lowering, antitumor, anti-infectant, skin repair, anti-aging, and cardiovascular preventive effects.[3] Amaranth seeds are the largest vegetal source of squalene, and major research work is focused on the variation in squalene content in amaranth oil. He and Corke, in an exhaustive study, stated the squalene content to range from 10.4 to 73.0 g/kg amaranth oil.[4]

Metabolite profiling has emerged as a significant method in food and pharmaceutical sciences in obtaining in-depth compositional information regarding the metabolome of any plant.[5] Metabolite profiling is used for identification and quantification of a selected number of known and unknown low-molecular-weight metabolites, generally related to a specific metabolic pathway(s).[6] GC-MS and NMR-based metabolic profiling could enable total and rapid analysis of the nutritional configuration.[7] However, no systematic work on nontargeted metabolite profiling of polar and nonpolar seed extracts of various *Amaranthus* cultivars using NMR spectroscopy and GC-MS has been done so far that would aim to identify and quantify metabolites of nutraceutical significance.

In the quest to unravel the nutritional potential of different cultivars, we understood the need of utilizing the metabolite profiling approach to obtain a comprehensive compositional study that has not been explored in amaranth seeds. The present study aims to investigate comprehensive polar and nonpolar metabolite profiling of six cultivars of *Amaranthus hypochondriacus* seeds using nontargeted GC-MS and NMR analyses coupled with multivariate statistical analysis via principal component analysis and to study the antioxidant potential of squalene content using various cellular-based antioxidant assays.

There is very limited research work on *A. hypochondriacus* seeds, and with an increasing number of food products and nutraceutical and health products processed from this functional food, its biological activity and mechanism need to be studied further. In this study, the components of triterpenes (squalene) in the seed of *A. hypochondriacus* were analyzed, and the effect of AM-3 (nonpolar extract with a high squalene content) on oxidative stress injury induced by hydrogen peroxide (H_2O_2) in human embryonic kidney 293 cells was studied for the first time, which is the theoretical basis for further utilization of *A. hypochondriacus* accumulation.

2. MATERIALS AND METHODS

2.1. Chemicals. The solvents utilized for extraction purposes of metabolites and HPLC investigation and the silica gel for chromatography were purchased from Merck Life Science Private Limited, Mumbai, India. GC-MS reagents methoxyaminehydrochloride, pyridine, and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), NMR reagents such as deuterium oxide (D_2O), deuterated chloroform ($CDCl_3$), and trimethylsilylpropionic acid (TSP), the standards of squalene for HPLC analysis, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) and butylated hydroxyl toluene (BHT) for the antioxidant assay were purchased from Sigma Chemical Company, USA.

2.2. Plant Materials and Their Processing. The experimental study was carried our on seeds of six different cultivars, namely, AM-1, AM-2, AM-3, AM-4, AM-5, and AM-6, of *A. hypochondriacus*, out of which three cultivars, namely, AM-1, AM-2, and AM-3, were procured from V.C.S.G. Uttarakhand University of Horticulture and Forestry, Ranichauri, Uttarakhand, India, and AM-4, AM-5, and AM-6 were

obtained from CSIR−National Botanical Research Institute (CSIR−NBRI), Lucknow. The seeds were lyophilized (Heto, Lyopro 6000, Denmark) until a fixed constant mass was obtained and were stored at −20 °C for extraction purposes.

2.3. Extraction of Metabolites. For nonpolar extraction, lyophilized *Amaranthus* seeds were powdered and 10 g of powdered seeds was extracted using hexane by Soxhlet extraction according to a method reported earlier.[8] The nonpolar extracts having secondary metabolites were stored at −20 °C until use. The polar extract was obtained by extracting lyophilized seeds (10 g) with MilliQ water using a mortar and pestle as reported earlier.[9] The filtered and concentrated polar extracts using a vacuum evaporator (EZ-2, Genevac) were stored at −20 °C for further analysis.

2.4. HPLC Analysis. Major biomarker squalene was analyzed using Waters reverse phase HPLC comprising a Waters 600 controller, Delta 600 solvent delivery system, rheodyne injector with a 20 μL loop, and Waters 2996 photodiode array (PDA) detector. The peak area was calculated with Waters Empowered 2.154 software. Chromatographic analysis was carried out in isocratic conditions using a μBondapak C18 reverse phase column (150 × 4.6 mm i.d., particle size 5 μm). Acetonitrile (100%) was used as the isocratic mobile phase at a flow rate of 1.0 mL min^{-1} as reported earlier.[10] Squalene in each sample was quantified by contrasting its chromatographic peak with the squalene reference standard at a wavelength of 195 nm.

2.5. GC-MS Analysis. Nonpolar metabolite characterization of the hexane extracts of amaranth cultivars was conducted via GC-MS analysis using 2,2,2-trifluoro-*N*-methyl-*N*-trimethylsilylacetamide (MSTFA) as the derivitizer to volatize the metabolites as described earlier.[11] The metabolites were identified as a TMS derivative with the help of a standard library (Replib, WILLY and NIST) compatible with the Automated Mass Spectral Deconvolution and Identification System (AMDIS32) and by matching the chromatograms with reference standards (Table S1). The peak areas were integrated by using Xcalibur2.1 software.

2.6. NMR Analysis. The quantitative estimation of polar metabolites was carried out on an 800 MHz NMR spectrometer (Bruker, Germany) fitted with a triple resonance cryoprobe. Polar extracts were dissolved in 500 μL of D_2O containing 0.03% (w/v) sodium salt of trimethylsilyl propionic acid (TSP), which were used as an internal standard for calibration and quantification of metabolites.[12] 2D-NMR experiments including $^{1}H-^{1}H$ COSY, $^{1}H-^{13}C$ HSQC, and $^{1}H-^{13}C$ HMBC were performed for the purpose of confirming chemical shift assignments, and data were integrated and evaluated as described previously.[13]

2.7. Statistical Analysis. Univariate analysis: The samples were extracted in triplicates, and the quantitative data was expressed as means ± standard deviations (SD). The comparison of quantified data of NMR and GC-MS analyses was determined statistically by one-way ANOVA using SPSS 11.5.0 (USA). The values were presented as mean ± SEM (standard error of the mean); metabolite differences among parts were considered statistically significant at $p < 0.05$. For the multivariate analysis, unsupervised principal component analysis (PCA) was utilized to explore the classification and discrimination in the polar and nonpolar metabolites of *Amaranthus* cultivars. PCA was performed on the pretreated normalization of the mean centered data of GC-MS and NMR using an Unscrambler X Software package (Version 10.0.1,

Table 1. Qualitative and Quantitative Variation Study of Nonpolar Metabolites Identified in Six Different Amaranth Cultivars Using GC-MS Analysis[a]

metabolites	AM-1	AM-2	AM-3	AM-4	AM-5	AM-6
2,4 di-*tert*-butyl phenol	$7.13 \pm 0.68^{b,c,d,e}$	$7.22 \pm 0.7.34^{f,g,h,i}$	$0.88 \pm 0.07^{j,k,l}$	$9.62 \pm 0.82^{m,n}$	18.64 ± 1.73^{o}	2.55 ± 0.21
hexadecane	$0.66 \pm 0.05^{c,e}$	$1.47 \pm 0.12^{g,h}$	$1.53 \pm 0.13^{j,l}$	4.21 ± 0.39^{m}	1.6 ± 0.18^{o}	4.99 ± 0.51
octadecene	0.9 ± 0.08^{c}	2.08 ± 0.22^{g}	2.55 ± 0.23^{j}	$5.01 \pm 0.48^{m,n}$	2.04 ± 0.19	2.31 ± 0.21
myristic acid	0.49 ± 0.04	0.43 ± 0.03	1.49 ± 0.12	0.42 ± 0.05	0.33 ± 0.02	0.37 ± 0.03
pentadecanoic acid	$3.7 \pm 0.35^{a,b,c}$	1.5 ± 0.13	0.97 ± 0.9^{l}	0.79 ± 0.07	1.92 ± 0.15	2.34 ± 0.25
palmitic acid	$8.24 \pm 0.78^{c,d}$	$7.93 \pm 0.75^{g,h}$	$8.1 \pm 0.79^{j,k}$	22.19 ± 2.1^{n}	19.90 ± 1.85^{o}	10.64 ± 1.0
docosene	ND	ND	ND	$1.67 \pm 0.13^{c,g,j}$	0.26 ± 0.02	$0.71 \pm 0.05^{e,l,l}$
oleic acid	$17.53 \pm 1.66^{a,b,c,d,e}$	$8.22 \pm 0.78^{g,h,i}$	$9.27 \pm 0.88^{j,k,l}$	$32.35 \pm 3.1^{m,n}$	29.21 ± 2.78^{o}	13.42 ± 1.14
linoleic acid	$6.75 \pm 0.72^{a,c,d,e}$	$22.55 \pm 1.89^{f,g,h,i}$	$8.43 \pm 0.79^{j,k,l}$	$28.05 \pm 2.54^{m,n}$	25.19 ± 2.39^{o}	16.04 ± 1.54
squalene	$29.53 \pm 2.5^{a,b,c,d,e}$	$37.96 \pm 3.62^{f,g,h,i}$	$54.32 \pm 5.32^{j,k,l}$	7.63 ± 0.73^{n}	6.01 ± 0.57^{o}	34.09 ± 3.13
pentacosanoic acid	1.77 ± 0.15^{e}	0.21 ± 0.02^{i}	1.4 ± 0.12^{l}	1.64 ± 0.17^{n}	2.04 ± 0.19	3.88 ± 0.36
α-tocopherol	$0.72 \pm 0.08^{b,c,d}$	$0.97 \pm 0.09^{f,g,h}$	ND	ND	ND	0.84 ± 0.07^{o}
stigmasterol	1.82 ± 0.13^{b}	2.12 ± 0.18	$3.96 \pm 0.35^{j,k}$	0.65 ± 0.06^{n}	0.71 ± 0.07^{o}	3.64 ± 0.04
3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propanoic acid	$1.64 \pm 0.14^{c,d}$	1.96 ± 0.21^{g}	$1.14 \pm 0.11^{j,k}$	3.82 ± 0.35	3.67 ± 0.34	1.93 ± 0.18

[a]Mean values $\pm$ SD of mg g^{-1} of the dry weight of seed samples; a–o denote statistical significance $p \leq 0.05$, i.e., a: AM-1 vs AM-2, b: AM-1 vs AM-3, c: AM-1 vs AM-4, d: AM-1 vs AM-5, e: AM-1 vs AM-6, f: AM-2 vs AM-3, g: AM-2 vs AM-4, h: AM-2 vs AM-5, i: AM-2 vs AM-6, j: AM-3 vs AM-4, k: AM-3 vs AM-5, l: AM-3 vs AM-6, m: AM-4 vs AM-5, n: AM-4 vs AM-6, o: AM-5 vs AM-66; ND: not detected.

CAMO, USA) in accordance with the protocol established previously.[14]

2.8. Determination of Antioxidant Activity Using Chemical and Cellular-Based Assays. *2.8.1. DPPH Free-Radical Scavenging Assay.* The antioxidant activity of the nonpolar extract of the six *Amaranthus* cultivars (AM-1−6) was evaluated for their antioxidant potential using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) method with butylated hydroxyl toluene (BHT) used as a positive control as well as a reference compound as reported in the literature previously.[9]

2.8.2. Cellular Antioxidant Assays. 2.8.2.1. Cell Culture. Human embryonic kidney cells (HEK293) were subcultured and routinely maintained in the aseptic control condition. During subculturing, 0.25% trypsin-EDTA solution was used to detach the adherent cells. HEK293 cells were seeded in a 96-well plate at a density of 1×10 cell/well and allowed to adhere for 24 h at 37 °C in a CO_2 incubator followed by various concentrations of treatment compound, after which the culture medium was replaced with a fresh medium, MTT solution was added to each well, and the plate was incubated for 4 h at a 37 °C in a CO_2 incubator, after which the medium was aspirated and the formed blue color compound was solubilized by adding DMSO/well for 30 min at 37 °C in a CO_2 incubator. Finally, the core solution intensity was quantified using a microplate reader at a wavelength of 570 nm.[15]

2.8.2.2. Effect of AM-3 on H_2O_2-Induced Oxidative Damage in HEK 293 Cells. Cells were resuscitated and cultured according to the experimental procedure in the cytotoxicity experiment of AM-3 on HEK293 cells. Cells were made into a cell suspension and cultured in 96-well plates. Cells were attached to the wall and then discarded. Further, 200 μL of deme medium containing 0.3 mmol/L H_2O_2 was added and cultured for 4 h to induce a cell oxidative damage model. After that, the medium was discarded again, the deme medium with a concentration of 50−1000 μg/mL AM-3 was added to culture for 48 h, and then, the OD value of each cell was measured at 494 nm according to the cytotoxicity experiment of AM-3 on HEK293 cells and the inhibition effect of AM-3 on oxidative damage induced by H_2O_2 in

HEK293 cells was observed by calculating the survival rate of cells after oxidative damage prevention by each concentration of AM-3.[16]

2.8.2.3. Morphological Observation of Cells. According to the effect of AM-3 on the oxidative damage of HEK293 cells induced by H_2O_2, the cells were cultured, oxidative damage was induced, and they were treated with AM-3 for 48 h. The morphology of cells in each group was observed under a microscope (EVOS M7000, Thermo Fisher Scientific, New York, USA).

2.8.2.4. Cellular ROS Detection Using the CELLROX Assay. Human embryonic cells (HEK293) were seeded onto a disc (22 mm) placed into a six-well plate and incubated for 6 h at 37 °C to adhere the cell on the surface. Cells were treated with a drug and incubated for 24 h. Hydrogen peroxide (H_2O_2) was added to cells at a concentration of 250 μM to induce oxidative stress, and incubation for 30 min at 37 °C was performed. A CellROX green reagent at a final concentration of 5 μM and Hoechst dye at a final concentration of 1 μg/mL were added to cells and incubated for 30 min at 37 °C. The cell culture medium was removed, and the cells were washed three times with PBS. The cells were fixed with 3.5% formaldehyde for 30 min at 40 °C. The fixing solution was removed, and cells were visualized at 485 and 360 excitation through a high content image analyzer. A negative control (without H_2O_2 treatment) and positive control (H_2O_2-treated) were used to detect the effect of drug on generation of reactive oxygen species.[17]

2.8.2.5. Lipid Peroxidation Detection Assay. A lipid peroxidation assay was performed using an Image-iT Lipid Peroxidation Kit. In brief, human embryonic kidney cells (HEK293) were seeded on a six-well plate with an inbuilt disc (20 mm) and incubated for 6 h at 370 °C. Cells were treated with a drug and incubated for 24 h. Cellular oxidative stress was induced through hydrogen peroxide, added to cells at a concentration of 250 μM, and they were incubated at 37 °C. Cells were stained through an Image-iT lipid peroxidation sensor component at a final concentration of 10 μM for 30 min at 37 °C, and nuclei were stained with Hoechst red dye. After incubation, the culture medium was aspirated, and cells were washed three times with PBS. Cells were fixed with 3.5%
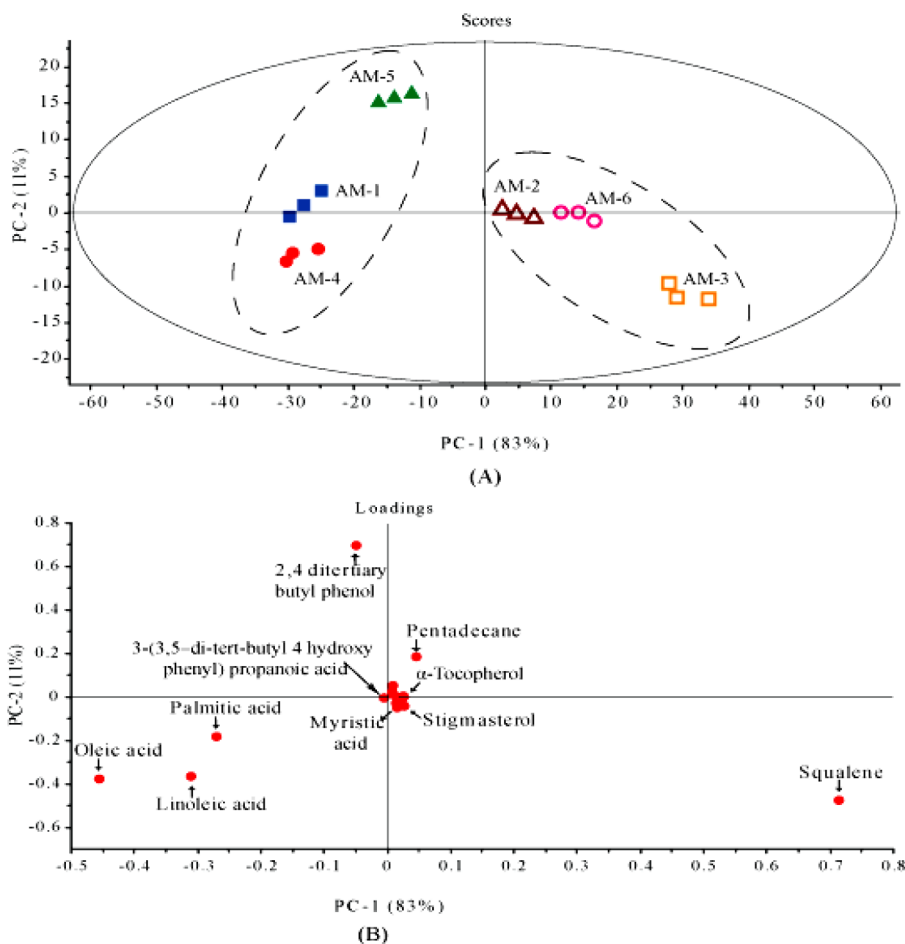
Figure 1. Stack plot of ^{1}H NMR spectra of polar extracts of six amaranth cultivars showing the variability in polar metabolites.

paraformaldehyde for 30 min at 40 °C. Afterward, high content imaging was performed at excitation 485 and 560 to visualize the effect of drug. Results were compared with the negative control and positive control.[18]

2.8.2.6. Determination of MDA, SOD, GSH, and CAT Levels in HEK293 Cells. According to the experimental operation of AM-3 on oxidative damage induced by H_2O_2 in HEK293 cells, the cells were cultured and treated with AM-3 for 48 h. Then, the MDA, SOD, GSH, and CAT levels in cells of each group were measured according to the kit instructions.[19]

2.8.3. Statistical Analysis. All experiments were carried out three times in parallel. The mean ± standard deviation (SD) was used to express the results. SAS9.1 statistical software was used to analyze the significant differences between groups of data at the level of $p < 0.05$. The analysis method was Duncan's multiple range test single factor analysis of variance (ANOVA).

3. RESULTS

3.1. Metabolic Profiling Using GC-MS. GC-MS-based metabolite profiling (Figure S1) of hexane extracts of six different amaranth cultivars resulted in identification of 14 metabolites consisting of four saturated fatty acids (myristic acid, pentadecanoic acid, palmitic acid, and pentacosanoic acid), two unsaturated fatty acids (oleic acid and linoleic acid), one dicarboxylic acid (oxalic acid), one alkane (hexadecane), two alkenes (octadecene and docosene), one triterpene (squalene), one vitamin (α-tocopherol), one phytosterol (stigmasterol), and two lipophilic phenols (butyl phenol and butyl propionate phenol), as shown in Table S1.

The concentration of metabolites varied significantly among hexane extracts as depicted by one-way ANOVA (Table 1). Squalene dominated over all of the metabolites in hexane extracts of seeds of *A. hypochondriacus*. The relative concentration of squalene ranged from 6% (wt) to 54% (wt), the lowest being in amaranth cultivar AM-5 and highest in AM-3. Occurrence of high concentrations of squalene in amaranth cultivar AM-3 (54.2%) suggests its use as a potential antioxidant agent.[20] Butyl phenol and butyl propionate phenol were the other metabolites detected in all the six cultivars of *A. hypochondriacus*.[21] A small quantity of pentadecanoic acid, an odd-chain saturated fatty acid that is an accepted biomarker of dairy fat intake, was detected in seeds of all the six cultivars of amaranth.[22]

Principal component analysis (PCA) was carried out to evaluate the GC-MS data of nonpolar extracts of six different cultivars, as shown in Figure 2A,B. It clearly demonstrated a notable cumulative variance of 94% between PC-1 and PC-2. The clusters observed in the score plot (Figure 2A) may be broadly classified into two groups. One group composed of cultivars, namely, AM-2, AM-3, and AM-6, while in the other group, cultivars AM-1, AM-4, and AM-5 formed another group separation. On examining the corresponding PCA scattered loading plot in Figure 2B, the metabolites responsible for the group separation among cultivars are mainly squalene, fatty acids (palmitic, oleic, and linoleic acids), and phenolics (butyl phenol and butyl propionate phenol). Squalene and lipophilic phenols, which are the major metabolites responsible for group separation in the score plot, are potent nutraceutical agents.[4]

3.2. Metabolic Profiling Using NMR. The polar extracts were analyzed using one-dimensional and two-dimensional

Figure 2. (A) PCA score plot obtained from the unsupervised PC analysis of GC-MS quantified values of nonpolar metabolites among six cultivars of *Amaranthus* seeds. (B) Loading plots for principal component 1.

NMR spectroscopy (Figure S2A,B), which enabled us to characterize the chemically diverse polar metabolites (Table S2). The stack plot of the ^{1}H NMR (Figure 1) of the six different cultivars conspicuously elaborated the presence and variation of primary metabolites through prominent signal intensities.

Our NMR study results, confirmed by an earlier reported literature and 2D ^{1}H−^{1}H COSY experiments, identified the presence of 33 polar metabolites composed of 14 amino acids, 7 sugars, 3 monocarboxylic acids, 1 dicarboxylic acid, 1 quaternary ammonium cation, 1 quaternary ammonium salt, 1 derivative of choline, 1 cyclohexane alcohol, 1 α-hydroxy acid, 2 glycosylated pyrimidines, and 1 alkaloid. One-way ANOVA showed significant quantitative variations in polar metabolites among cultivars (Table 2).

The PCA score plots showed two significant cluster formations. The two groups so formed consisted of AM-3, AM-2, and AM-6 in one group, and the other composed of AM-1, AM-4, and AM-5. The PC-1 vs PC-2 score plot of polar extracts of seeds (Figure 3A) along with corresponding loading (Figure 3B) from different cultivars revealed a clear variance of 85%. The corresponding PCA loadings indicated that such group separation emerged due to the differentiating metabolites such as arginine, tryptophan, lysine, phenylalanine, glutamic acid, GABA, sugars like glucose, trehalose, and maltose, betaine, and myoinositol.

3.3. HPLC-Based Quantitative Determination of Squalene.
A quantitative variation in squalene content among different cultivars of *Amaranthus* was carried out using HPLC following an earlier reported method.[10] The squalene content varied significantly among cultivars ranging from 1.61 to 4.72 mg g^{-1} dry weight of amaranth seeds, the lowest being in AM-5 and the highest being in AM-3 (Table 3). A similar trend of squalene content among cultivars was observed in the GC-MS analyzed data (Table 1).

3.4. Antioxidant Potential Estimation on the Chemical and Cellular Level.
3.4.1. DPPH Free-Radical Scavenging Activity. The DPPH free-radical scavenging capacity measured by the IC$_{50}$ value of the hexane extracts of the six different *Amaranthus* cultivars ranged from 430.12 to 730.65 μg mL^{-1} (Table 4), with the lowest (high antioxidant activity) being in amaranth AM-3 and highest (low antioxidant activity) in cultivar AM-5 (Figure S3). Linear regression analysis was utilized to understand the association between the squalene content and the antioxidant capacity (IC$_{50}$ values) of the extracts (Figure S4).

A negative correlation between the squalene content of nonpolar extracts and their IC$_{50}$ values ($y = -100.04x + 903.95$, Pearson coefficient $r = -0.9679$) further explains the positive correlation between the squalene content and its antioxidant activity. Hence, we identified squalene as the major bioactive metabolite responsible for the potent antioxidant capacity of amaranth.[23]

Table 2. ^{1}H NMR-Based Quantitative Analysis of Polar Metabolites from Six *Amaranthus* Cultivars[a]

metabolites	AM-1	AM-2	AM-3	AM-4	AM-5	AM-6
acetate	0.82 ± 0.72[a,b,e]	4.49 ± 0.37[f,g,h]	7.55 ± 0.63[j,k,l]	1.09 ± 0.08[n]	0.49 ± 0.53[o]	4.11 ± 0.37
alanine	0.31 ± 0.03[a,b,c]	11.25 ± 1.2[f,g,h,i]	2.46 ± 0.22[k]	1.67 ± 0.18[m]	8.08 ± 0.71[o]	1.10 ± 0.95
arginine	2.54 ± 0.19[a,b,c,d,e]	22.99 ± 0.27[f,g,h,i]	7.50 ± 0.78[j,l]	5.09 ± 0.43[m,n]	7.76 ± 0.64[o]	26.14 ± 0.21
betaine	5.68 ± 0.51[a,b,e]	9.21 ± 0.84[f,g,h]	3.66 ± 0.28[j,k,l]	6.03 ± 0.57[n]	7.10 ± 0.75	8.69 ± 0.91
β-fructose	17.87 ± 1.73[b,c,d,e]	16.76 ± 1.48[f,g,h,i]	4.77 ± 0.41[j,k]	11.64 ± 1.13[m,n]	9.19 ± 0.81[o]	3.07 ± 0.27
β-galactose	4.66 ± 0.53[a,b,c,e]	42.52 ± 4.37[f,g,h,i]	10.10 ± 0.92[j,k]	6.74 ± 0.65[m,n]	3.90 ± 0.46[o]	9.68 ± 0.91
β-glucose	62.65 ± 0.59[a,b,c,e]	37.35 ± 2.91[g,h,i]	35.30 ± 3.42[j,k,l,]	65.12 ± 6.61[m,n]	62.17 ± 0.58[o]	8.38 ± 0.78
choline	3.65 ± 0.34[b,e]	2.10 ± 0.18[i]	0.85 ± 0.07[l]	2.64 ± 0.22[n]	2.31 ± 0.19[o]	5.85 ± 0.61
formate	0.09 ± 0.008[a,b,c]	7.25 ± 0.67[f,g,h,i]	2.27 ± 0.17[k,l]	3.41 ± 0.31[m,n]	0.01 ± 0.001[o]	N.D
gaba	0.80 ± 0.07[a,b,c,d,e]	17.51 ± 1.43[f,g,h,i]	10.45 ± 1.021[j,k,l]	4.24 ± 0.36[n]	3.72 ± 0.28[o]	14.88 ± 1.27
glutamine	6.66 ± 0.53[e]	6.39 ± 0.54[i]	5.62 ± 0.48[l]	6.54 ± 0.67[n]	7.50 ± 0.61[o]	23.49 ± 2.18
glycine	6.90 ± 0.72[d]	6.94 ± 0.55[h]	3.57 ± 0.33[k]	7.62 ± 0.63[m]	10.06 ± 0.95[o]	3.13 ± 0.24
Gpc	N.D.	2.98 ± 0.25[a,g,h,i]	N.D	N.D	N.D	4.27 ± 0.38[e,l,n,o]
isobutyric acid	2.13 ± 0.17[a,c,e]	22.92 ± 2.07[f,g,h,i]	3.24 ± 0.29[l]	4.56 ± 0.37	3.23 ± 0.27[o]	5.68 ± 0.43
isoleucine	1.27 ± 0.11[a,b]	9.82 ± 0.91[f,g,h,i]	3.77 ± 0.40[k,l]	1.86 ± 0.14	1.36 ± 0.08	0.71 ± 0.06
lactate	17.35 ± 1.68[a,b,c,d,e]	26.37 ± 2.21[f,g,h,i]	30.46 ± 0.26[j,k,l]	N.D.	38.45 ± 3.54[m,o]	1.15 ± 1.03[n]
leucine	0.32 ± 0.027[a]	5.30 ± 0.49[f,g,h,i]	2.16 ± 0.16	1.20 ± 0.10	1.06 ± 0.08	0.68 ± 0.05
lysine	9.32 ± 0.86[a,b,c,d,e]	80.49 ± 7.86[f,g,h,i]	40.38 ± 3.94[j,k,l]	17.71 ± 1.64[n]	15.72 ± 1.31[o]	72.58 ± 7.11
maleic acid	N.D.	7.60 ± 0.68[a,g,h]	7.29 ± 0.74[b,j,k]	1.06 ± 0.95[c,m,n]	N.D.	8.86 ± 0.85[o]
maltose	68.74 ± 7.2[a,b,c,d,e]	57.75 ± 5.39[f,g,h,i]	73.85 ± 7.12[j,k,l]	91.12 ± 90.89[n]	92.02 ± 9.11[o]	36.01 ± 3.21
methionine	2.97 ± 2.84[a,b,e]	19.34 ± 1.84[g,h,i]	19.39 ± 1.82[k,l]	1.18 ± 0.97[m,n]	4.71 ± 4.3	6.58 ± 6.45
myoinositol	61.00 ± 5.74[a,b,c,d,e]	55.33 ± 5.3[f,g,h,i]	33.72 ± 3.2[j,k,l]	72.64 ± 7.11[m,n]	79.07 ± 7.83[o]	22.89 ± 2.41
phenylalanine	3.86 ± 0.36[a,b,c,d,e]	49.49 ± 5.1[f,g,h,i]	62.67 ± 6.72[k,l]	19.25 ± 1.67[m,n]	0.72 ± 0.06[o]	67.09 ± 6.56
proline	N.D.	20.55 ± 2.1[a,f,g,h,i]	15.81 ± 1.5[b,j,k,l]	N.D.	N.D.	5.96 ± 0.53[e,n,o]
raffinose	1.34 ± 0.01[a,b,e]	22.42 ± 2.23[f,g,h,i]	5.33 ± 0.46[j,k,l]	3.48 ± 0.29[n]	1.74 ± 1.64[o]	10.98 ± 1.06
sucrose	12.06 ± 1.1[a,b,c,d,e]	24.00 ± 2.37[f,g,i]	6.57 ± 0.62[j,k,l]	28.77 ± 2.53[m,n]	23.40 ± 2.15[o]	15.52 ± 1.34
trehalose	66.12 ± 6.48[a,b,c,e]	54.58 ± 5.24[f,g,h,i]	43.64 ± 4.16[j,k,l]	81.43 ± 7.83[m,n]	65.91 ± 6.49[o]	13.31 ± 1.19
trigonelline	6.31 ± 0.59[a,b,c,d,e]	24.02 ± 2.23[f,g,h,i]	13.89 ± 1.34[k,l]	12.62 ± 1.14[m,n]	0.36 ± 0.03[o]	9.30 ± 9.11
tryptophan	1.03 ± 0.08[a,b,c,d,e]	42.24 ± 4.13[f,g,h,i]	16.68 ± 1.57[j,k,l]	11.12 ± 1.1[m,n]	14.16 ± 1.45[o]	33.49 ± 3.21
tyrosine	0.70 ± 0.06[a,b,e]	24.27 ± 2.41[f,g,h,i]	6.33 ± 0.58[j,k]	1.12 ± 0.07[n]	0.56 ± 0.45[o]	8.36 ± 0.81
uracil	1.30 ± 0.11[a,b,c,e]	25.06 ± 2.26[f,g,h,i]	10.80 ± 0.94[k]	10.48 ± 1.02[m]	3.02 ± 0.3[o]	12.62 ± 1.18
uridine	12.02 ± 1.24[a,b,c,d]	42.19 ± 4.17[f,g,h,i]	38.11 ± 3.79[j,k,l]	16.62 ± 1.47[m]	8.75 ± 0.93[o]	13.96 ± 1.42
valine	0.63 ± 0.57[a,b]	8.77 ± 0.83[g,h,i]	9.03 ± 0.85[j,k,l]	1.62 ± 0.14	1.20 ± 0.11	0.61 ± 0.05

[a]Mean values ± SD of mg g^{-1} of the dry weight; a−o denotes statistical significance $p \leq 0.05$, i.e., a: AM-1 vs AM-2, b: AM-1 vs AM-3, c: AM-1 vs AM-4, d: AM-1 vs AM-5, e: AM-1 vs AM-6, f: AM-2 vs AM-3, g: AM-2 vs AM-4, h: AM-2 vs AM-5, i: AM-2 vs AM-6, j: AM-3 vs AM-4, k: AM-3 vs AM-5, l: AM-3 vs AM-6, m: AM-4 vs AM-5, n: AM-4 vs AM-6, o: AM-5 vs AM-6, ND: not detected.

3.4.2. Toxic Effect of AM-3 Nonpolar Extract on HEK293 Cells. When HEK293 T cells were treated with the AM-3 nonpolar extract at concentrations of 50, 100, 200, 400, 600, 800, and 1000 μg/mL, compared with HEK293 T cells that were not treated with AM-3, AM-3 at concentrations of 50−1000 μg/mL did not significantly affect the proliferation of HEK293 T cells, and the survival rate of HEK293 T cells in this concentration range was more than 95% (Figure 4A). Therefore, 100, 300, and 600 μg/mL AM-3 nonpolar extracts were used as sample concentrations for further tests.

3.4.3. Oxidative Stress Injury Induced by H_2O_2 in HEK293 Cells. The survival rate of HEK293 cells treated with H_2O_2 was significantly lower than that of HEK293 cells without H_2O_2 treatment, and the survival rate was only 23.7% (Figure 4B). The survival rate of HEK293 cells increased with an increase in the concentration of the AM-3 nonpolar extract. When concentration of AM-3 was 600 μg/mL, the survival rate of the HEK293 cells increased to 82.6%.

3.4.4. Morphological Changes Induced by H_2O_2 in HEK293 Cells. After cell culture, the normal HEK293 cells were found to be abundant in quantity, complete in cell structure, abundant in cytoplasm, and orderly in cell arrangement in a microscopy field (Figure 5); after being treated with H_2O_2, the number of HEK293 cells was decreased compared with the number of cells without H_2O_2 treatment. The cell morphology was severely atrophied, the cell structure was changed, and the cell damage was severe. AM-3 could obviously improve the abnormal cell structure caused by H_2O_2 and could restore the cell structure and quantity. With an increase in the AM-3 concentration, the recovery of cells was better.

3.4.5. Cellular ROS Scavenging Activity Detection Using the CELLROX Assay. Generation of ROS induces oxidative stress in cells, leading to cellular damage and cell death. At the cellular level, the antioxidant potential of AM-3 (highest squalene content) was evaluated by detecting the reduction of cellular ROS induced in cells by H_2O_2 treatment. For that purpose, the fold change in mean fluorescence intensity (MFI) of ROS in all the cells (negative control, BHT-treated, and AM-3-treated) was monitored. An increase in fold change in MFI compared to healthy cells indicated oxidative stress in cells due to ROS accumulation. Figure 6A clearly demonstrates that there was a substantial increase in fold change in MFI of the negative control, suggesting the presence of high oxidative stress in these cells caused by large accumulation of ROS due to H_2O_2 treatment. The cells when pretreated with nonpolar
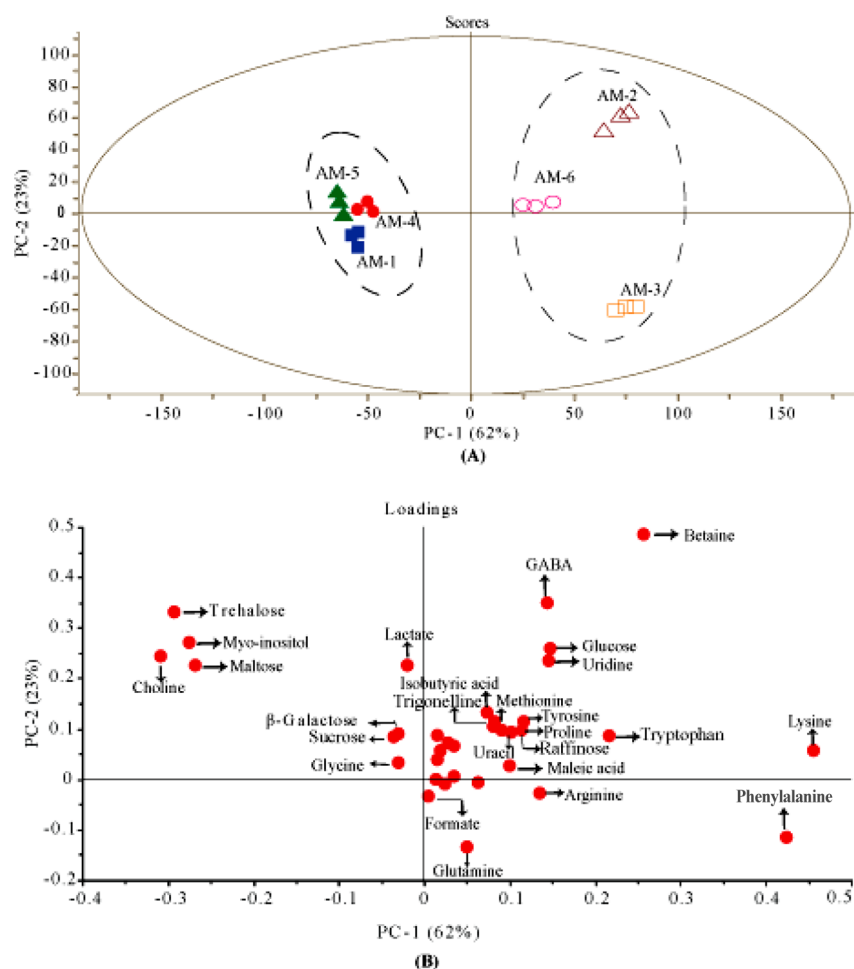
Figure 3. (A)PCA score plot obtained from the unsupervised PC analysis of ^{1}H NMR quantified values of polar metabolites among six cultivars of *Amaranthus* seeds. (B) Loading plots for principal component 1.

Table 3. HPLC-Based Quantitative Estimation of the Squalene Content[a]

Amaranthus cultivars	squalene mg/g dry weight of amaranth seeds
AM-1	2.47 ± 0.18^b
AM-2	2.57 ± 0.19^f
AM-3	$4.72 \pm 0.33^{j,k}$
AM-4	2.69 ± 0.22
AM-5	1.61 ± 0.14^o
AM-6	3.14 ± 0.28

[a]Mean values $\pm$ SD of mg g^{-1} dry weight; values followed by different letters, a−o, denote statistical significance $p \leq 0.05$ ($n = 3$).

Table 4. IC$_{50}$ Values of *Amaranthus* Nonpolar Extracts Showing DPPH Radical Scavenging Capacity[a]

	IC$_{50}$ values (μg/mL)
squalene	23 ± 14
AM-1	661 ± 47^b
AM-2	625 ± 39^f
AM-3	$430 \pm 22^{j,k}$
AM-4	693 ± 58
AM-5	730 ± 64°
AM-6	571 ± 31

[a]Mean values $\pm$ SD of mg gm^{-1} dry weight. Values followed by different letters, a−o, denote statistical significance $p \leq 0.05$ ($n = 3$).

extract AM-3 at concentrations of 100, 300, and 600 μM showed visible reduction in fold change in MFI, suggesting inhibition of ROS, leading to diminished oxidative stress in these cells (Figure 6B). Thus, amaranth AM-3 shows promising ability to prevent oxidative stress induced in cells by scavenging cellular ROS, making amaranth a strong antioxidant candidate.

3.4.6. Lipid Peroxidation Assay. H$_2$O$_2$-induced cellular ROS cause cell membrane destruction in a cell through a lipid peroxidation reaction, which occurs between the membrane lipids and the generated peroxide free radicals. This detrimental effect can also lead to cell death.[24] The cell image obtained from the high content imaging system showed that lipid peroxidation in the cells led to the shifting of peak florescence emission from 590 nm (red) to 510 nm (green), as shown in Figure 7A,B. The ratio of the red/green florescence was used to estimate the lipid peroxidation level in cells. The healthy cells (positive control) had maximum red signals and showed a high 590/510 ratio. Upon H$_2$O$_2$ treatment, in the non-pretreated cells (negative control), the ratio was much lower and the image showed maximum green signals, suggesting massive lipid peroxidation. In contrast, AM-3-pretreated cells at a concentration of 300 μM showed a higher 590/510 ratio and more red signals than green signals as compared to nontreated cells (Figure 7A). The histogram shown in Figure 7B clearly shows the inhibition of membrane
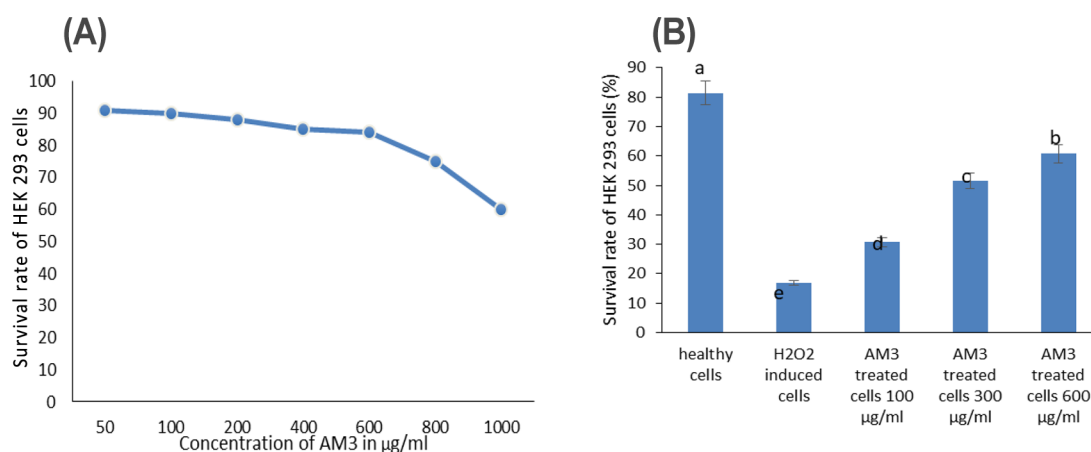
Figure 4. (A)Toxic effect of AM-3 (maximum squalene content) nonpolar extract at different concentrations on HEK293 T cells. Values presented are the mean ± standard deviation. (B) Effect of AM-3 (maximum squalene content) nonpolar extract at different concentrations on H_2O_2-induced oxidative damage HEK293 cells. Values presented are the mean ± standard deviation. Different letters indicate significant differences from each other at the level of $p < 0.05$ according to Tukey's honest significant difference. Normal: untreated HEK293 cells; model: hydrogen peroxide-induced oxidative damage HEK293 cells.
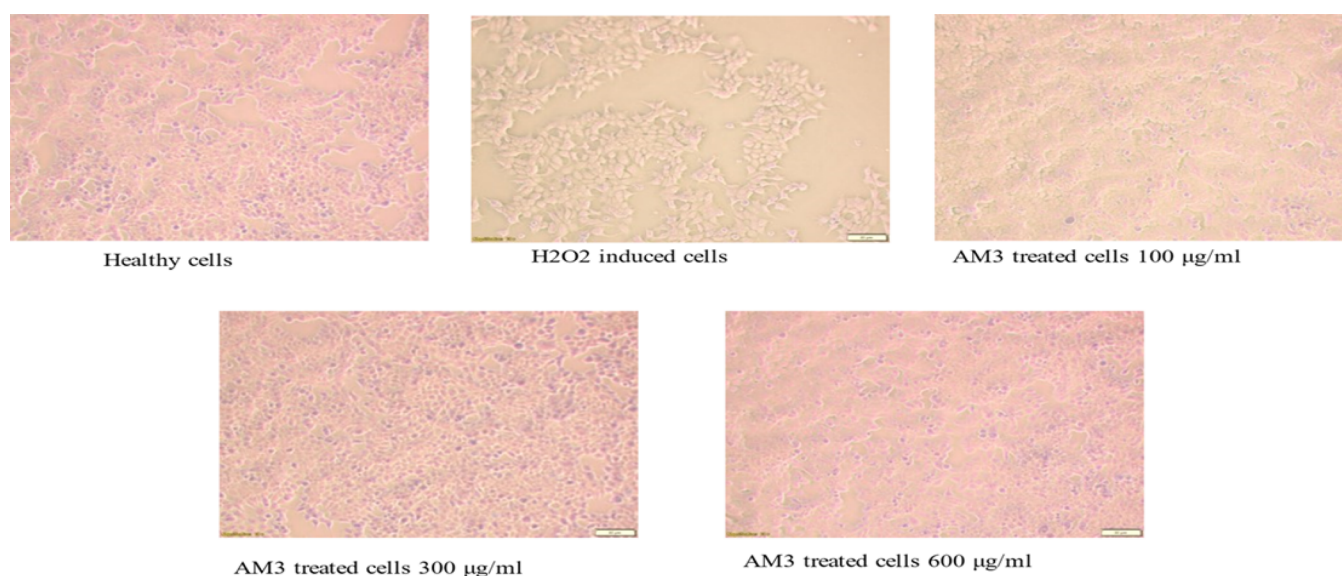
Figure 5. Morphological observation of H_2O_2 damage to HEK293 cells. Healthy cells: untreated HEK293 cells; hydrogen peroxide-induced oxidative damage HEK293 cells.

lipid peroxidation by AM-3 nonpolar extract at various concentrations, thus suggesting its potent cellular antioxidant activity.

3.4.7. Effects of AM-3 on the Levels of MDA, SOD, GSH, and CAT in HEK293 T Cells Treated with H_2O_2. The MDA level in HEK293 cells treated with H_2O_2 was significantly higher than that in cells not treated with H_2O_2 ($p < 0.05$), while the SOD, GSH, and CAT levels were significantly lower ($p < 0.05$, Table 5). The concentration of AM-3 at 100, 300, and 600 μg/mL decreased the MDA level and increased the levels of SOD, GSH, GSH PX, and CAT in the cells treated with H_2O_2. Among them, the concentration of AM-3 at 600 μg/mL restored the MDA, SOD, GSH, and CAT levels in the cells closest to those in normal HEK293 cells (Table 5).

4. DISCUSSION

In our present study, we have tried our best to present for the first time a comprehensive metabolic profiling of *Amaranthus* seeds to explore its complete quantitative composition. Through a nontargeted GC-MS and NMR-based metabolomics study, we could achieve the snapshot of the complete metabolome of the seeds. Grain amaranth has emerged as a complete functional food with a plethora of macro- and micronutrients and also the largest source of squalene after shark liver oil. *A. hypochondriacus* seeds contain a rich spectrum of both saturated and unsaturated fatty acids such as palmitic (saturated fatty acid), α-linoleic also known as ALA (poly-unsaturated fatty acids), and oleic acid (monounsaturated fatty acid). Oleic and linoleic acids were the other major fatty acids present in the nonpolar extracts of amaranth seeds. The concentration of alpha linoleic acid ranged from 6.75 to 28.05%, the lowest being in AM-1 and highest in AM-4. As it is well known, ALA is frequently suggested to minimize the risk of cardiovascular disease, obesity, lifestyle diseases, and cancer. The percent peak areas of α-tocopherol, a potent lipid soluble antioxidant,[25] were 0.72, 0.97, and 0.84%, in AM-1, AM-2, and
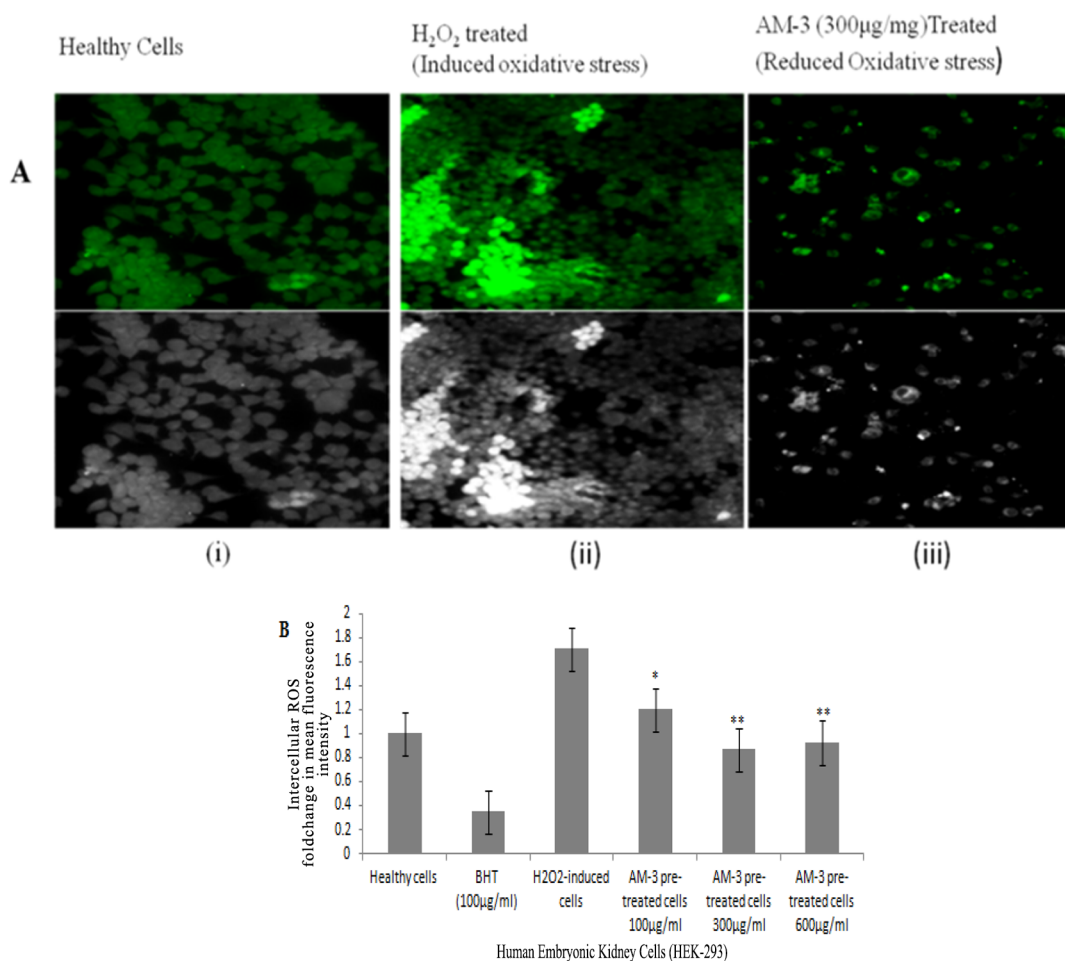
Figure 6. (A) Effect of AM-3 (maximum squalene) nonpolar extract (at 300 μg/mg) on scavenging cellular ROS (antioxidant potential) induced in human embryonic cells (HEK293) by H_2O_2. (B) Intercellular ROS scavenging activity of various concentration of AM-3 on HEK cells.

AM-6 respectively. Butyl phenol and butyl propionate phenol were the other metabolites detected in all six cultivars of *A. hypochondriacus*. The percent peak area of butyl phenol ranged from 0.88% in AM-3 to 18.64% in AM-5 with an average of 7.67% (34). 2,4-Di-*tert*-butyl phenol isolated from sweet potato is a potent antioxidant agent that has been reported to have a protective effect against amyloid-beta peptide-induced neurotoxicity.[21] The nonpolar extract of *Amaranthus* seeds shows to possess many essential nutrients in good quantity, providing high health benefits for human consumption.

Our NMR study results concurred with the earlier reported literature and 2D ^{1}H$-^1$H COSY experiments and identified the presence of 33 polar metabolites. Three essential amino acids, namely, lysine, tryptophan, and methionine, which are limited in available cereals and legumes, were detected in all six cultivars of *Amaranthus*. The literature reports that lysine seems to aid in calcium absorption and plays a crucial part in the synthesis of collagen, a substance essential for bones and connective tissues such as skin, tendons, and cartilage.[25] The lysine content in seeds ranged from 9.32 mg g^{-1} in AM-1 to 80.49 mg g^{-1} dry weight seeds in AM-2. Methionine is another essential amino acid detected in amaranth seeds, known for its various health properties.[26] A maximum of 19.39 mg g^{-1} methionine in AM-3 and a minimum of 1.18 mg g^{-1} dry weight of seeds in AM-4 were quantified in our study. Arginine is yet another semi-essential amino acid that was detected in

seeds of all six cultivars. The concentration of arginine ranged from 2.54 mg g^{-1} in AM-1 to 26.14 mg g^{-1} dry weight of seeds in green amaranth. An earlier work has reported that arginine deficiency in preterm infants results in various health problems such as hyperammonemia, as well as cardiovascular, pulmonary, neurological, and intestinal dysfunction.[27] Myoinositol was the other major metabolite of nutraceutical potential[28] whose concentration ranged from 22.89 mg g^{-1} in AM-6 to 79.07 mg g^{-1} dry seeds in AM-5 with an average of 54.10 mg. Gamma amino butyric acid (GABA) is a nonprotein amino acid crucial for the metabolism of the brain. It functions as the primary inhibitory neurotransmitter in the brain.[29,30] GABA is known to be able to lower neuronal excitability by slowing various brain processes, aiding anxiety, depression, epilepsy, insomnia, and a wide range of neurological disorders. Additionally, GABA exhibits antihypertension, antidiabetes, anticancer, antioxidant, anti-inflammation, antimicrobial, and antiallergy properties as significant biological activities. Furthermore, GABA is a potent liver, kidney, and intestinal protective agent against toxin-induced damage.[31] This crucial metabolite can be obtained from a variety of dietary sources, and our study has reported a significant amount of GABA content in the polar extracts of *Amaranthus*, namely, AM-2 (17.51 mg/g) and AM-6 (14.88 mg/g).

Amaranthus seeds are the largest vegetal source of squalene and have 2.4−8.0% of squalene in grains.[32] The occurrence of a high concentration of squalene in amaranth cultivar AM-3
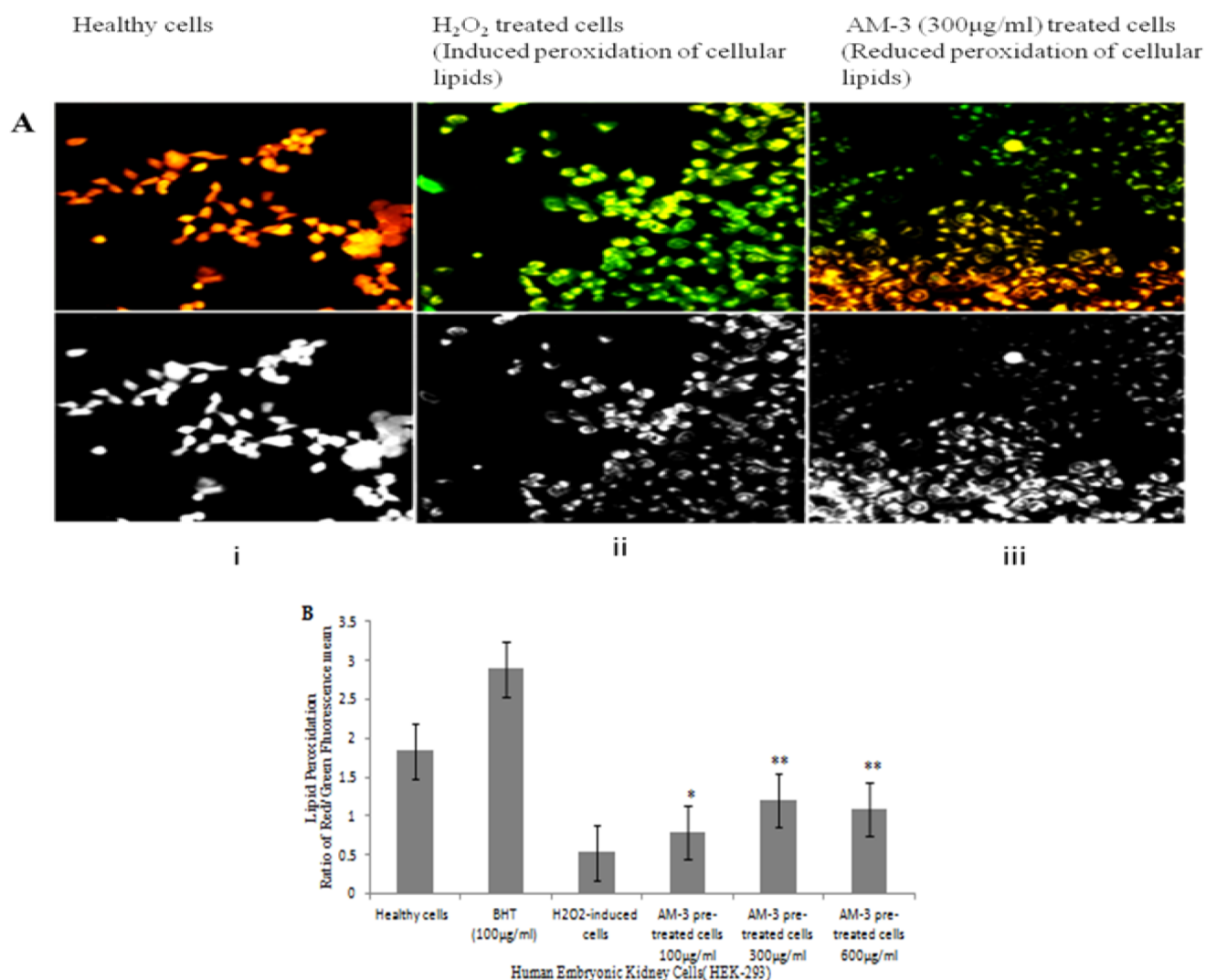
Figure 7. (A) Effect of AM-3 (maximum squalene content) nonpolar extract (at 300 μg/mL) on preventing lipid peroxidation (antioxidant potential) in human embryonic cells (HEK293). (B) Histogram showing the antilipid peroxidation activity of various concentrations of AM-3.

Table 5. MDA, SOD, GSH, and CAT Levels of H_2O_2 Damage HEK293 Cells[a]

groups	SOD relative activity (%)	CAT relative activity (%)	GSH relative activity (%)	MDA relative activity
healthy cells	0.036 ± 0.008^a	35.1 ± 1.15^a	1.1 ± 0.06^a	0.521 ± 0.11^e
H_2O_2-induced cells	0.017 ± 0.002^e	14.3 ± 0.97^e	0.46 ± 0.07^e	5.14 ± 0.17^a
AM-3-treated cells 100 εg/mL	0.025 ± 0.004^d	26.65 ± 5.24^d	1.3 ± 0.04^d	2.37 ± 0.15^b
AM-3-treated cells 300 εg/mL	0.030 ± 0.006^c	30.58 ± 0.74^c	1.4 ± 0.04^c	0.815 ± 0.09^c
AM-3-treated cells 600 εg/mL	0.031 ± 0.007^b	40.49 ± 0.74^b	1.19 ± 0.04^b	0.743 ± 0.08^d

[a]Values presented are the $\pm$ standard deviation. Different letters indicate significant differences from each other at the level of $p < 0.05$ according to Tukey's honest significant difference. Normal: untreated HEK293 cells; model: hydrogen peroxide-induced oxidative damage HEK293 cells

(54.2%) in Table 3 suggests its use as a potential antioxidant agent. In an initial DPPH-based assay, we screened the six nonpolar extracts of *Amaranthus* extracts and found AM-3 with the highest squalene content to show maximum antioxidant potential. That gave us the lead to proceed with AM-3 extract for various cellular-based antioxidant assays. In this study, the antioxidant effect and mechanism of *A. hypochondriacus* in vitro were preliminarily studied.

After the occurrence of oxidative stress, normal metabolism and increments of tissue cells are affected. In serious cases, a large number of cells die, and proliferation slows or even stagnates. At the same time, abnormal oxidative stress leads to the destruction of normal cell structure, apoptosis of cells, and then abnormal cell death. A serious decline affects human health and even threatens life. Reducing the degree of apoptosis caused by oxidative stress can protect the tissues and cells and resist the attack of diseases on the body.[36] In this study, AM-3 has been proven to inhibit abnormal cell death and apoptosis caused by oxidative stress in vitro; thus, it protects the cells.

MDA is recognized as a marker of oxidative stress. MDA released from the cell membrane can react with protein and nucleic acids, causing cross-linking polymerization and making normal synthesis of the protein impossible. MDA can also

cause abnormal structure and function of the cell membrane, thus causing an abnormal physiological state of the body.[38] SOD and CAT are antioxidant enzymes in vivo. MDA is highly cytotoxic and can inhibit antioxidant enzymes in vivo. Oxidative stress caused by abnormal expression of these indicators is involved in the pathogenesis of many clinical diseases.[38] GSH is also an active peptide with a good antioxidant effect, which can effectively regulate the oxidation balance of the body and inhibit the damage caused by oxidative stress.[39] The results of this study also showed that AM-3 can interfere with the abnormality of oxidation-related indexes after H_2O_2 treatment of cells, and thus, it reduces the cell damage caused by oxidative stress and plays a role in protecting the normal cells.

Squalene is a poly-unsaturated triterpene hydrocarbon found in amaranth seeds, known for its excellent antioxidant effect. Squalene is not only used as an antioxidant in the food industry, but it also has anti-inflammatory effects.[40,41] *Amaranthus* seeds contain a high amount of squalene. Their direct interaction constitutes the biological activity of AM-3, which confers good ability to resist oxidative stress and the potential to prevent and treat other diseases. This triterpene produces stable semiquinone free radicals through the reaction of phenolic hydroxyl group with free radicals, thus terminating the chain reaction of free radicals, which is the main mechanism for triterpenes to scavenge free radicals.[42] At the same time, triterpenes combine with the cell membrane in the form of a hydrogen bond to protect the unsaturated bond of the cell membrane from contacting with free radicals, thus playing the role of antioxidant protection.[43] In this study, AM-3 may also play a role in avoiding the human embryonic kidney 293 cell damage by hydrogen peroxide.

5. CONCLUSIONS

The nontargeted metabolomics approach was found to be a useful tool for identifying various metabolites of nutraceutical significance from the comparative study of polar and nonpolar seed extracts of six cultivars of *Amaranthus*. The PCA of the polar and nonpolar seed extracts revealed clear differentiation of the cultivars in two groups and their variable metabolic concentrations.

HPLC-based quantification of squalene showed the highest content of squalene in AM-3 nonpolar extract. An important result derived from our study showed that the maximum squalene (0.47%)-containing cultivar showed the highest antioxidant capacity with an IC_{50} value of 430 μg mL^{-1}. The results of correlation studies showed that the squalene content was positively correlated with antioxidant activity, showing that this triterpene is the major bioactive responsible for the antioxidant potential of amaranth. This was further proven by the strong antioxidant activity of AM-3 at the cellular level. AM-3 exhibited strong intercellular ROS scavenging and membrane lipid peroxidation inhibition in HEK-293 cells. It also showed ability to reduce oxidative stress in cells by reducing levels of MDA, SOD, GSH, and CAT in HEK293 T cells. Thus, it is a preferred choice for a strong natural antioxidant.

Substantial quantity of biologically active metabolites of nutraceutical significance and strong cellular-based antioxidant potential indicated the promising use of amaranth extracts in the development of targeted nutraceuticals and dietary supplements. In this study, the results showed that the high squalene-containing AM-3 nonpolar extract could protect HEK293 cells from oxidative stress by regulating oxidation, apoptosis, and inflammation. This study preliminarily verified the effect of AM-3, but its antioxidative effect and mechanism in vivo need to be studied further. In the future, an in-depth study is needed to assess the role of AM-3 in the intervention of various diseases through its antioxidant effect.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05597.

> GC-MS chromatogram, ^{1}H−^{1}H NMR COSY spectrum of polar extract of *A. hypochondriacus* seeds, correlation graph between IC_{50} values and the corresponding squalene contents, DPPH radical scavenging IC_{50} values of all extracts, squalene, and BHT, mass fragmentation of GC-MS identified nonpolar metabolites, and chemical shift of ^{1}H NMR characterization of polar metabolites (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Annie Gupta − *CSIR−National Botanical Research Institute, Lucknow, UP 226 001, India*; Email: anniegupta0601@gmail.com

Sunil Kumar Panda − *Menovo Pharmaceuticals Limited, Shanghai 200000, People's Republic of China*; Email: pandasunil17@gmail.com

Authors

Gaurav Deep Singh − *Radha Govind University, Ramgarh 226001, India*

Arti Gautam − *CSIR−National Botanical Research Institute, Lucknow, UP 226 001, India*

Tusha Tripathi − *CSIR−National Botanical Research Institute, Lucknow, UP 226 001, India*

Amit Kumar Taneja − *Jiangxi Fushine Pharmaceutical Co., Ltd., Jingdezhen 245608, People's Republic of China*

Brahma N Singh − *CSIR−National Botanical Research Institute, Lucknow, UP 226 001, India*

Raja Roy − *Centre of Biomedical Research, Formerly Known as Centre of Biomedical Magnetic Resonance, Sanjay Gandhi Postgraduate Institute of Medical Sciences Campus, Lucknow, UP 226014, India*

Om P. Sidhu − *CSIR−National Botanical Research Institute, Lucknow, UP 226 001, India*

Arun Bhatt − *Department of Crop Improvement, VCSG Uttarakhand University of Horticulture & Forestry, Tehri Garhwal, Uttarakhand 249199, India*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.3c05597

Notes

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