Cancer Chemopreventive Potential and Chemical Profiling of *Euphorbia abyssinica* Endowed with Docking Studies

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**ABSTRACT:** Chemical profiling of both fruit and aerial part extracts of *Euphorbia abyssinica* via ultra-performance liquid chromatography−mass spectrometry (UPLC-MS) showed them to be a rich source of diverse compounds. A total of 39 compounds in both extracts including flavonoids and phenolic compounds were identified as predominant metabolites. The antioxidant activity of both extracts was evaluated using three different in vitro assays (DPPH, ABTS, and FRAP assays). The *E. abyssinica* fruit extract demonstrated more potent activity compared to the aerial part extract (IC$_{50}$ of 85.1 ± 1.07 and 562.3 ± 1.01 μg/mL, respectively) in the DPPH assay. Furthermore, using ABTS and FRAP assays, the antioxidant capacities of the fruit extract were 1063.03 ± 37.8 and 1476.5 ± 95.6, respectively, calculated as μM Trolox equivalent/mg extract. One of the existing markers for cancer chemoprevention is the induction of phase II detoxifying enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1), which plays a vital role in cytoprotection against oxidative damage. The extracts were assessed to test their chemopreventive potential via NQO1 enzyme induction. The methanolic extract of fruits demonstrated a concentration-dependent increase in the cancer chemopreventive marker enzyme NQO1 at the protein expression level in a murine hepatoma cell line (Hepa1c1c7). The interaction with Kelch-like ECH-associated protein 1 (KEAP1) is an essential transcription factor that controls the expression of the NQO1 enzyme. The demonstrated induction of NQO1 by the fruit extract is consistent with a molecular docking study of the dereplicated compounds on the KEAP1 target. Among the dereplicated compounds, hesperidin, naringin, and rutin have been established as promising inducer compounds for the chemopreventive marker NQO1. Our results highlight the *E. abyssinica* fruit extract as a future chemopreventive lead.

**INTRODUCTION**

Cancer is a growing global health problem. According to a new survey by the World Health Organization (WHO), there are currently more than 10 million cancer cases around the world each year. The field of cancer chemoprevention, which is concerned with preventing the development of cancer, is currently evolving in an attempt to counteract the steady increase in cancer morbidity and mortality worldwide. Reactive oxygen species cause oxidative stress, resulting in cell damage or eventually changing the genetic material in a normal cell into a transformed one. Activation of detoxifying enzymes can also combat carcinogenesis and protect cells from the effects of ultimate carcinogens. Various natural and synthetic chemopreventive agents are utilized in cases when there is an elevated risk of developing cancer or to prevent recurrence of cancer after treatment. One of the existing markers for cancer chemoprevention is the induction of phase II detoxifying enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1). An important chemoprevention strategy is the blocking of carcinogenic insult damage to DNA (carcinogenesis initiation stage). The two-electron reduction of quinones to less hazardous water-soluble hydroquinones has been demonstrated by NQO1, providing protection to cells against oxidative damage caused by quinones. NQO1 has been linked to a chemopreventive and anticarcinogenic action as a two-electron reductase. In this approach, induction of NQO1 is hypothesized to become a cytoprotection strategy. Nuclear factor erythroid 2-related factor 2 (NRF2) is a major regulator of cytotoxic genes that regulate the production of detoxifying and antioxidative enzymes such as NQO1. Kelch-like ECH-associated protein 1 (KEAP1) is a component of the Cullin 3 (CUL3)-based E3 ubiquitin ligase complex and regulates the stability and accumulation of NRF2. Compre-
Comprehensive genomic analyses have identified mutations and other changes in the KEAP1 or NRF2 genes in different types of cancer. Inhibition of KEAP1-NRF2 protein-protein interaction can impair NRF2 degradation, resulting in the accumulation of newly synthesized NRF2 and its translocation to the nucleus, where it induces transcription of a battery of
antioxidative and cytoprotective genes, leading to activation of the cellular defense system. Therefore, NRF2 and KEAP1 have been investigated as potential targets for cancer therapy. Several plant-derived chemical compounds have shown to be promising chemopreventive agents. *Euphorbia* is a diverse genus belonging to the Euphorbiaceae family with a substantial...
percentage, mainly from Africa and Madagascar. Desert candle (Euphorbia abyssinica) has been traditionally used to treat intestinal worms, dysentery and fungal infection on the head, external injury, venereal diseases, and neck cancer. Various extracts of E. abyssinica stem bark have been reported to have broad-spectrum antifungal activity. In addition, the plant latex has been proven to have antifungal and antibacterial effects. The root extract demonstrated antimalarial activity against Plasmodium berghei infection in mice. An ointment prepared from the plant latex showed antibacterial and antiparasitic activities for the treatment of skin diseases. Chemically, the latex of the plant contained 8(R)-hydroxy-dec-3(E)-en-oic acid, β-sitosterol, lupeol, oleanolic acid, and β-sitosterol-3-O-glucoside. Regarding the current literature, several studies have investigated the chemical profiles of different Euphorbia species and correlated the documented bioactivities to the abundant secondary metabolites present in plant extracts. Despite the diversity of chemical and biological studies on the genus Euphorbia, there are few studies on E. abyssinica and no systematic study on its chemopreventive potential and chemical profiling. Therefore, the present study provides a comparative insight into the antioxidant and cancer-preventive potential of E. abyssinica fruits and aerial parts by tracing the induction of well-established cytoprotective enzyme NQO1 in relation to their metabolite make-up. Also, the molecular docking of the binding affinity of all dereplicated compounds inside active sites of KEAP1 was examined to show possible mechanisms of action responsible for the chemopreventive activity.

RESULTS AND DISCUSSION

UPLC-MS Chemical Profiling. The chemical profiles of both the E. abyssinica fruit extract and aerial part extract (EFE and EAE, respectively) were characterized by UPLC-MS. As shown in Table S1, a total of 39 compounds including 25 flavonoids, 10 phenolic compounds, 3 coumarins, and 1 fatty acid were identified in both extracts. EFE was characterized by the presence of 10 flavonoids (4, 17, 22, 23, 25, 28, 29, 34–36) and one phenolic compound (6) that were absent in EAE (Figures 1–3). The chemical composition of E. abyssinica has not been well defined in the literature; however, flavonoids and phenolic compounds are very distinct compounds in the genus Euphorbia. It is noteworthy that this is the first comprehensive study addressing the metabolomics profile of E. abyssinica.

Antioxidant Activity. Reactive oxygen species scavenging activity has demonstrated its effect on a variety of biological processes, including reducing oxidative stress and thereby...
Table 1. In Vitro Antioxidant Activity of Euphorbia abyssinica

| Sample                | DPPH (IC₅₀) (μg/mL) | ABTS (μM Trolox equivalent/mg extract) | FRAP (μM Trolox equivalent/mg extract) |
|-----------------------|---------------------|--------------------------------------|---------------------------------------|
| Fruit extract (EFE)   | 85.1 ± 1.07         | 1063.03 ± 37.8                       | 1476.5 ± 95.6                         |
| Aerial part extract   | 562.3 ± 1.01         | 343.3 ± 13.7                         | 447.5 ± 10.4                          |
| Trolox                | 24.4 ± 0.87         |                                      |                                       |

“All data are presented as mean ± SD.

The endogenous antioxidant NQO1 enzyme is one of the essential two-electron antioxidant enzymes belonging to the NAD(P)H:quinone oxidoreductase (quinone) family. It is an essential antioxidant that can use NADH or NADPH as a reducing dehydrogenase. NQO1 is a protein that is upregulated in response to oxidative stress. NQO1 is a protein that is upregulated in response to oxidative stress. In several neoplastic models, this transcriptionally controlled phase II enzyme is involved in redox-sensitive chemoprotection. Western blot is regarded as a reliable and effective technique of choice for the detection of a specific protein since the approach relies on a unique antigen–antibody interaction upon protein separation into distinct bands. The study findings demonstrated the potential of E. abyssinica fruit extract (EFE) as a promising candidate in the treatment of oxidative stress-related carcinogenesis via upregulation of NQO1 protein expression.

Molecular Docking Study. Kelch-like ECH-associated protein 1 (KEAP1) was targeted to evaluate the chemopreventive potential of dereplicated compounds to pinpoint some possible mechanism of action. The interaction with KEAP1 is a fundamental transcription factor for the helix–loop–helix agent, NRF2, which controls the expression of phase II cytoprotective enzymes such as NQO1 that detoxifies cancer and protects against oxidative stress (Figure 5).

The induction of several antioxidant enzyme systems provides cytoprotection in response to oxidative stress. NQO1 is a protein that is upregulated in response to oxidative stress. In several neoplastic models, this transcriptionally controlled phase II enzyme is involved in redox-sensitive chemoprotection. Western blot is regarded as a reliable and effective technique of choice for the detection of a specific protein since the approach relies on a unique antigen–antibody interaction upon protein separation into distinct bands. The study findings demonstrated the potential of E. abyssinica fruit extract (EFE) as a promising candidate in the treatment of oxidative stress-related carcinogenesis via upregulation of NQO1 protein expression.

Cancer Chemopreventive Activity. The treatment of Hepa1c1c7 cells with increasing concentrations of the E. abyssinica fruit extract (EFE) triggered an increase in the protein expression of the chemopreventive marker NQO1, as revealed by western blotting analysis (Figure 4). However, the aerial part extract did not demonstrate any activity.

NAD(P)H:quinone oxidoreductase 1 (NQO1) is an antioxidant enzyme that belongs to the NAD(P)H dehydrogenase (quinone) family. It is an essential two-electron reductase that can use NADH or NADPH as a reducing cofactor. The endogenous antioxidant NQO1 enzyme is one of the most consistently and robustly inducible genes among members of the cytoprotective protein family against oxidative stress. NQO1 is generally expressed in various tissues under transcriptional regulation by the KEAP1/NRF2 pathway that mediates upregulation of NQO1, which has been extensively used as a chemopreventive biomarker. Regulation of NQO1 has been hypothesized to protect cells from oxidative damage due to NQO1’s ability to decrease ROS free radicals produced by the unstable production of hydroquinone.

According to the results of the molecular docking study, 23 compounds demonstrated lower binding energies for the reference ligand (4′-bromoflavone); however, none of the tested compounds exceeded the binding energy of the cocrystallized ligand (Table S2). The cocrystallized ligand N,N′-naphthalene-1,4-diybis(4-methoxybenzenesulfonyamide) showed a binding energy of −10.7 kcal/mol, while 4′-bromoflavone (CID: 1686), which was used as the reference compound, demonstrated a binding energy of −8.0 kcal/mol. Three compounds hesperidin (CID: 10621), naringin (CID:...
Figure 6. Binding modes and interaction types of (A) N,N′-naphthalene-1,4-diybis(4-methoxybenzenesulfonamide) (cocrystallized ligand), (B) hesperidin, (C) naringin, (D) rutin, and (E) 4′-bromoflavone (reference ligand) with KEAP1. Green dotted lines represent hydrogen bonding; other interactions are of hydrophobic nature.

The chemopreventive marker NQO1.

The observed results of in silico modeling according to previous reports establish the three compounds (hesperidin, naringin, and rutin) as promising inducer compounds for the KEAP1 protein target, posing them as candidates for further in vitro and in vivo future studies as cancer prevention drug leads.

### MATERIALS AND METHODS

**Plant Material.** *E. abyssinica* fruits and aerial parts were collected from Ahmed Alaa botanical garden, Toukh, Qalyubia, Egypt in March 2019. They were authenticated by Tereze Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture, Giza, Egypt. A voucher specimen (March 21, 2019) was placed at the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

**Preparation of Plant Extracts.** Fresh plant materials, fruits and aerial parts (1 kg, each), were extracted separately with methanol using an ultraturrax homogenizer until exhaustion followed by evaporation under vacuum (Buchi 210, Switzerland) to obtain the dried residue and stored at 4 °C for further investigations.

**Chemicals and Reagents.** HPLC grade acetonitrile, methanol, and water (Thermo Fisher Scientific Inc., Dublin, Ireland) were used for UPLC-MS analysis. Other chemicals of analytical grade used in the present study were obtained from Sigma-Aldrich Chemical Co. (Ireland). For the chemopreventive study, murine hepatoma cells (Hepa1c1c7) were obtained from (ATCC). All reagents and chemicals for cell culture and bioassays were purchased from Sigma-Aldrich (Steinheim, Germany) or Lonza (Verviers, Belgium). Plasticware was purchased from Greiner Bio-One (Frickenhausen, Germany). Primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Thermo Fisher Scientific (anti-NQO1 cat. no. PA5-21290, anti-β-actin cat. no. MA5-15739, goat anti-rabbit cat. no. 31460, and goat anti-mouse cat. no. 31430).

**General Experimental Methods. UPLC-MS Analysis.** Natural extracts are commonly composed of hundreds to thousands of metabolites, whereby the bioactivity of natural extracts can be represented by synergism between several metabolites. However, the isolation of each single compound from natural extracts is not always attainable due to the complex chemistry and the presence of most secondary metabolites from crude natural extracts. Profiling via ultra-
performance liquid chromatography (UPLC) coupled with high-resolution MS was utilized to characterize secondary metabolites that could be intermediates in the chemopreventive activity of *E. abyssinica* fruit and aerial part crude extracts. UPLC with high-resolution mass spectrometry offers several advantages including high separation efficiency and excellent resolution with relatively short analysis time. Metabolite profiling of both the *E. abyssinica* fruit extract (EFE) and the aerial part extract (EAE) was performed according to a previously described method using a Waters Acquity UPLC system (Waters, Manchester, U.K.) hyphenated with an Orbitrap-type, HRMS (Exactive, Thermo Fisher, Bremen, Germany). Metabolites were identified by comparing retention time and MS data (accurate mass and fragmentation pattern in positive ionization mode, with the error, adjusted 0–10 ppm) in the current literature. In addition, certain compounds have been identified using the database of “Dictionary of Natural Products”, CRC.

**Antioxidant Activity.** Three in vitro assays were used to assess the antioxidant activity of EFE and EAE: 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), and ferric reducing antioxidant power (FRAP).

**DPPH Assay.** The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical assay was performed using the method described by Boly et al. Samples were prepared in DMSO at concentrations of 1000 and 100 μg/mL to determine a range in which inhibitory concentration 50 (IC_{50}) was present. A stock solution of 100 μM Trolox was prepared in methanol from which seven concentrations were prepared including 50, 40, 30, 20, 15, 10, and 5 μM. Briefly, in a 96-well plate (n = 6), 100 μL of the freshly prepared DPPH reagent (0.1% in methanol) was mixed with 100 μL of the sample, and the reaction was incubated at room temperature for 30 min. in the dark. The resulting decrease in DPPH color intensity was measured at 540 nm at the end of the incubation time. The results were recorded using a FluoroStar Omega microplate reader.

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\% \text{ inhibition} = \left( \frac{\text{average absorbance of blank}}{\text{average absorbance of test}} \right) \times 100
\]

The IC_{50} value was determined using GraphPad Prism 5 after converting the concentrations to their logarithmic value and selecting the nonlinear inhibitor regression equation (log (inhibitor) vs a normalized response—variable slope equation). The assay was performed on microplates using the method by Arnao et al. with minor modifications. The samples were dissolved in DMSO and prepared at concentrations of 1 mg/mL for EAE and 0.4 mg/mL for EFE. A 1 mM Trolox stock solution in methanol was prepared, followed by five serial dilutions at concentrations of 600, 500, 400, 300, 200, 100, and 50 μM. In brief, 192 μg of ABTS were dissolved in distilled water and transferred to a 50 mL volumetric flask, and then the volume was completed with distilled water. One milliliter of the previous solution was added to 17 μL of 140 mM potassium persulphate and the mixture was left in the dark for 24 h. Subsequently, 1 mL of the reaction mixture was completed up to 50 mL with methanol to obtain the final ABTS dilution used in the assay. In a 96-well plate (n = 4), 190 μL of the freshly prepared ABTS reagent was mixed with 10 μL of the sample and the reaction was incubated at room temperature for 120 min. in the dark. The decrease in ABTS color intensity was measured at 734 nm at the end of the incubation time. The results were recorded using a microplate reader FluoStar Omega. The antioxidant activities were represented as μM Trolox equivalent/mg extract using the linear regression equation driven from Trolox’s linear dose–inhibition curve. Data were depicted as mean ± SD.

**FRAP Assay.** The ferric reducing ability assay was performed using the method described by Benzie et al. with minor modifications to be performed in microplates. The samples were prepared at a concentration of 1 mg/mL in DMSO. A Trolox stock solution in methanol was prepared at a concentration of 5 mM, and 10 serial dilutions were prepared at 4000, 3000, 2000, 1000, 800, 600, 400, 200, 100, and 50 μM. Briefly, 10 μL of the sample was mixed with 190 μL of the newly prepared TPTZ reagent (300 mM acetate buffer (pH = 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM FeCl₃, in a ratio of 10:1:1 (v/v/v, respectively) in a 96-well plate (n = 3), and the reaction was incubated at room temperature for 30 min. in the dark. The blue color generated was detected at 593 nm at the end of the incubation period. The results were recorded via a FluoroStar Omega microplate reader. The ferric reducing ability of the samples was presented as μM Trolox equivalent/mg extract using the linear regression equation extracted from the linear dose–inhibition curve of Trolox. Data were expressed as mean ± SD.

**Cancer Chemoprevention Assessment.** 

**Cell Culture.** In a humidified incubator (Sartorius CMAT, Germany, 5% CO₂/95% air), a monolayer culture of a murine hepatoma cell line (Hepa1c1c7) was maintained in alpha-modified Minimum Essential Medium Eagle (α-MEME) supplemented with 10% (v/v) heat- and charcoal-inactivated fetal bovine serum, 2 mM l-glutamine, 100 mM penicillin, and 100 μg/mL streptomycin. The cells were sub-cultured with a trypsin EDTA solution at around 80% confluence.

**Assessment of the Induction of NQO1 in Hepa1c1c7 Cells.** Briefly, cells (3 × 10⁵ cells/mL) were seeded onto 6-well plates and allowed to adhere and form monolayer overnight. For an additional 24 h, the monolayers were treated with either vehicle (concentration 0.1% v/v DMSO) or plant extracts (100 μg/mL). In addition, 4′-bromomolvane (10 μM) was used as a standard for NQO1 induction. The monolayers were rinsed with ice-cold Dulbecco’s PBS (2 mL/well) after aspiration of treatment media. The cells were scraped and transferred to labeled microcentrifuge tubes in ice-cold lysis buffer (25 mM Tris-Cl, pH 7.4, 250 mM sucrose, and 5 mM FAD). After that, the cell suspensions were sonicated on ice for 5 s (20% amplitude). After centrifuging the sonicates at 4 °C, the supernatants were aliquoted and kept in a freezer at −80 °C until analysis.

**NQO1 Western Blotting.** Hepa1c1c7 overnight cultures (seeded at zero time as 3 × 10⁵ cells/well of 6-well plates) were treated with vehicle (0.1% DMSO), positive control (4′-bromomolvane), or extracts (final concentrations of 6.25, 25, and 100 μg/mL). Treatment was performed for 24 h after which cell lysates were prepared. The samples (fixed amount of 25 μg of total proteins/lane as determined using a Thermo Nanodrop spectrophotometer) were resolved under denaturing conditions by electrophoresis (SDS-PAGE) on 10% acrylamide/bisacrylamide gel (150 V for 1.5 h). After that, the resolved proteins were transferred to nitrocellulose membranes at 100 V for 90 min. After blocking for 1 h at 25 °C in 5% non-fat milk in Tris-buffered saline with 0.1%
Tween 20 (TBST), the membranes were probed overnight (4 °C) with primary antibodies against NQO1 and β-actin (Thermo Fischer Scientific) with gentle shaking. After 3 × 5 min washes in TBST, probing with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies started for 1 h at 25 °C. The membranes were then washed (3 × 5 min.) in TBST before being developed with enzyme chemiluminescence (ECL, Pierce). A chemiluminescence imager (UVP, U.K.) was used to digitally visualize protein bands on the developed membranes.

**Molecular Docking Study.** The crystal structure of the Kelch-like ECH-associated protein 1 (KEAP1) domain in complex with N,N′-naphthalene-1,4-diylbis(4-methoxybenzenesulfonamide) (PDB ID: 4I0Q, resolution: 1.97 Å) was downloaded from the Protein Data Bank. The three-dimensional (3D) structures of the chemical compounds were downloaded from the PubChem database. AutoDockTools was used for the generation of the input files of protein structures, cocrystallized ligand, and studied chemical compounds. All ligands were processed with Open Babel v2.4.1. AutoDockVina software was used for molecular docking of the studied compounds with the investigated proteins. Grid boxes were selected, with sizes not exceeding 27 000 Å³, and the studied compounds with the investigated proteins. Grid parameter in case of using small boxes. Docking methodology was validated by redocking the cocrystallized ligand. Ligplot was used for visualization and analysis of the interactions of docked compounds with corresponding proteins.

**Notes**

The authors declare no competing financial interest.

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