Original Research Article

In vitro cytotoxic and antimicrobial effects of a novel peroxysesquiterpene glucoside from the rhizomes of Cyperus rotundus L (Cyperaceae)

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Abstract

**Purpose:** To study the antimicrobial and cytotoxic potential of a novel 3,9-peroxysesquiterpene-15-O-glucoside from Cyperus rotundus rhizomes, against HeLa cell line and selected strains of microorganisms.

**Methods:** The rhizomes were macerated with methanol and fractionated with n-hexane, chloroform, ethyl acetate and butanol. High-performance liquid chromatography (HPLC) was performed together with chemical analysis of the fractions. The 3,9-peroxysesquiterpene-15-O-glucoside was purified through column chromatography of the ethyl acetate fraction, and its purity was determined via reverse-phase HPLC. Structural elucidation was done with Infrared (IR), proton nuclear magnetic resonance (1H NMR), carbon-13 nuclear magnetic resonance (13C NMR), and mass spectrometry (MS) spectroscopic analyses.

**Results:** The isolated compound exhibited bactericidal and fungicidal activities against S. aureus and C. albicans at concentration, respectively, in the range of 32 – 100 µg/mL, while MTT assay results showed the cytotoxicity of the compound against eukaryotic (HeLa) cell line (IC50, 88.32 µg/mL).

**Conclusion:** The isolated metabolite from the methanol extract of C. rotundus rhizome exhibits bactericidal, fungicidal, and cytotoxic potential. However, further studies are required to ascertain its suitability for use as a therapeutic agent.

**Keywords:** Terpenoids, Column chromatography, Spectroscopy, Antimicrobial, Cytotoxicity, Cyperus rotundus

INTRODUCTION

The contribution of natural metabolites to drug discovery is evident in the existence of numerous lead molecules of biological significance, like terpenoids, with interesting bioactivities. The plant Cyperus rotundus L. (Cyperaceae, CR) is an indigenous herb from the Iraqi-Kurdistan Region. It is a known source of several metabolites, including novel terpenoids. These metabolites are valuable in drug discovery due to their unusual biological activities such as antibacterial, antifungal and cytotoxic properties. They are abundant in many plant species, and...
their diverse chemical composition is attributed to variations in the enzymatic pathways involved in their biosynthesis [1].

*Cyperus rotundus* L. expresses these terpenoid metabolites, but despite its folk medicinal uses for the treatment of GIT disturbances, fever, malaria, and bronchitis, the plant has not received much research attention. Extract of *C. rotundus* rhizomes exerts diuretic, analgesic, antibacterial, antifungal, and cytotoxic properties. Studies on the rhizomes have revealed the presence of important metabolites like sesquiterpenes, sterols, and flavonoids [2]. Although numerous studies have investigated the biological properties of *C. rotundus* rhizomes [3, 4], the antioxidant, antimicrobial and cytotoxic bioactivities were based on the crude extract (CE), and were not attributed to specific isolated component(s).

The process of drug discovery focuses on generation of lead compounds with bioactive properties in different aspects of medicine e.g. antimicrobial compounds and cytotoxic agents. Microbial resistance to chemotherapeutic drugs requires the discovery of new agents with the ability to inhibit the growth of resistant strains. Metabolites capable of targeting microbes at sites not utilized by the known antimicrobials are in high demand, and plants provide an unlimited reservoir of these metabolites [5].

Screening for molecules with cytotoxic potential is a widespread practice in drug discovery because of the high mortality rates from cancer around the world [6]. The trend in drug discovery is to screen natural products for new hits that can enhance apoptosis in mutated cells. The present study was carried out to determine the cytotoxic effect of extract of rhizomes of *C. rotundus* by investigating to identify isolated bioactive metabolites with promising antimicrobial and cytotoxic potentials.

**EXPERIMENTAL**

**Materials**

Rhizomes of *Cyperus rotundus* L. (Cyperaceae (CR)) were harvested, dried, ground to coarse powder and stored in freezer until use. The "non-fastidious" microorganisms *Escherichia coli* (ATCC No. 8739), *Staphylococcus aureus* (ATCC No. 6538) and *Candida albicans* (ATCC No. 10231) purchased from Medya-Med labs (Erbil, Iraq) were used in biological activity assessment. Mueller-Hinton agar (38 g/L D.W.) and broth media were prepared in accordance with *Bergey’s Manual of Systematic Bacteriology*. Dulbecco’s Modified Eagle Media (DEMEM), and Fetal Bovine Serum (FBS) were purchased from TransBionovo (China). Human endothelial HeLa cell line (EA.hy926) bought from Fine Test (China) was used for cytotoxicity assay of the isolated compound. All other chemicals and reagents used in this assay were of analytical grade.

**Plant extraction and metabolite isolation**

The plant was authenticated at Kurdistan Botanical Foundation, and a voucher specimen was deposited in the medicinal herbarium at College of Pharmacy, University of Sulaimani, Iraq. The dried and crushed rhizome (1.5 kg) was extracted using 1 L methanol in a shaking water bath set at 100 rpm at 30 °C. The solvent was replaced daily until exhaustive extraction was achieved. The methanolic extract was concentrated under vacuum in a rotary evaporator to yield 195 g of crude extract of CE. The extract was then resuspended in adequate warm distilled water, and then partitioned in triplicate in equal volumes of n-hexane (Hex), chloroform (CHCl₃), ethyl acetate (EtOAc), and n-butanol (BtOH). Each of the eluted fractions and the mother liquor (83.5 g) were dried under vacuum at 30 °C [7].

Next, 2 mg from the crude extract and the fractions from partitioning were dissolved in suitable solvent and subjected to TLC using Hex: EtOAc: Formic acid at volume ratio of 10:1:0.3 [8]. To detect terpenoids, the plates were sprayed with Liebermann-Burchard reagent and heated to 100 °C for 10 min.

The EtOAc fraction was flash-chromatographed using Hex: EtOAc (50 to 0:0 to 50) over silica gel, resulting in the collection of 70 x 3-mL fractions [7]. The collected fractions were subjected to TLC using pet. ether: EtOAc: formic acid at volume ratio of 10:1:0.3, and the spots were detected under UV-254, 302, and 365 nm. Fractions showing identical Rf profiles were pooled and dried under vacuum and reduced temperature. Two sequential column elutions using Sephadex LH-20 were carried out with pet. Ether: EtOAc (50 to 0:0 to 50), with collection of 50 x 3-mL-fractions [24, 25]; and CHCl₃: MeOH (0 to 10:10 to 0), with collection of 20 x1-mL fractions. In each elution process, the subfractions were chromatographed over TLC using the same environment stated above, and spots with identical Rf-values were combined. To identify the bioactive metabolites, antimicrobial property was assessed using disc diffusion assay for the CE extract and all pooled sub-fractions, as described in the materials section [8].
Single spots were recovered with preparative thin layer chromatography (PTLC) using silica gel G and pet. ether: EtOAc: formic acid (10:1:0.3, v:v:v) on glass plates [8]. Spots were scratched and recovered with adequate acetone. The solvent was removed and the crystals were obtained. The purity of the sample was confirmed with HPLC (Waters e 2695, USA Alliance HPLC systems) using reverse-phase C-18 (150 mm × 4.6 mm .5 μm) column at 30 °C. The solvent system used for analysis was acetonitrile: water (60:40, v:v) at a flow rate of 1 mL/min, and injection volume of 50 µL. Detection was at wavelengths of 254, 302, and 365 nm [9]. After HPLC confirmation, the crystals were lyophilized and subjected to structural elucidation using spectrometric methods (FT-IR, 1H NMR, and MS).

The presence of terpenoids in the crude extract and the sub-fractions was detected using the Salkowski method for essential oils [10] and Liebermann-Burchard reagent, followed by heating at 100 °C for 10 min [11]. The presence of peroxide was detected by adding 1 mL test solution to a freshly prepared solution of 0.1 g NaI in 1 mL glacial acetic acid [12].

Spectroscopy analysis

Fourier Transmission-IR spectrum (Perkin-Elmer) (FT/IR) was performed using KBr pellets (Vmax in cm⁻¹), and the data was interpreted using Spectrum-A software. The 1H NMR analysis was performed by dissolving 2 mg of the test sample in MeOH, and the spectrum recorded on Avance-III 400 MHz NanoBay. The assay conditions were as follows: spectrometer frequency (SF) = 400.1500 MHz, acquisition time (AQ) = 4.0894465 sec, relaxation delay (D1) = 1.0 sec, pulse width = 45°, spectral width (SWH) = 8012.820 Hz, digital resolution = 0.3 Hz, and Fourier transform (TE) size = 298.1K. Chemical shifts were calculated in ppm relative to D-glucose molecule (CDCl₃, δH 7.24 and δC 77.0). The data were presented and recorded using Topspin 3.2 software. The 13C NMR analysis was carried out by dissolving 2 mg of material in DMSO. Chemical shifts were recorded as parts per million (SF = 100.635 MHz, AQ = 0.917 sec, D1 = 1.0 sec, SWH = 35714.285 Hz, and line broadening = 1.00 Hz). Mass spectroscopy analysis was carried out using ESI, and the sample was injected directly with a continuous flow rate of 10 μL/mL for a total run time of 1 min. Ion detection was done at positive mode (m/z +1), using the volume ratio of 50:50 for carrying diluent methanol: water. The concentration of the sample solution was adjusted to 5 μg/mL. The following detection parameters were used: DP = 40, EP = 10, and IS = voltage 5500. Using ion source spray gas 30, the data was interpreted with Analyst Software version 1.6.3.

Determination of antimicrobial effects of the compounds

Minimum inhibitory concentration (MIC) was determined according to the previously established method of Clinical and Laboratory Standards Institute (CLSI), with Mueller-Hinton broth microdilution method at a final microbial concentration of 5 × 10⁵ cells/mL [14]. Serial dilutions of test compounds, standards, blank and positive control were prepared at concentrations of 1, 2, 4, 8, 16, 32, 64, and 100 µg/mL, in 96-well microplates. The plates were incubated overnight in a-200 rpm shaking incubator (Stuart Orbital Incubator) at 35 ± 2 °C. Microbial growth was determined using a microplate reader at 600 nm. All experiments were performed in triplicate. The antibacterial and antymycotic activities of the isolated compounds were compared to those of gentamicin and fluconazole, respectively. The lowest concentration that inhibited microbial growth was considered as MIC. Check to evaluate minimum bactericidal concentration (MBC), a loop from each concentration showing inhibition of the microorganism was inoculated on Mueller Hinton agar plates (MHAP), and incubated overnight at 36 ± 2 °C. Dilutions with the lowest concentrations that showed no microbial growth were considered as MBCs [33].

Cytotoxicity and MTT assay

Cells were seeded and incubated in a culture flask in DMEM containing 10% FBS, with ampicillin and kanamycin (1 μL/mL and 0.5 µL/mL, respectively) in a humidified 5 % CO₂ incubator at 37 °C. Sub-culturing for refreshment of the culture was performed on alternate days. The cells were treated with serial dilutions of the test compounds (CE and 3,9-peroxysesquiterpene-15-O-glycoside at concentrations of 20, 40, 80, 160 and 200 µg/mL). The blank comprised medium without cells, while the positive control included cells with media. All inoculation processes were performed on sterile 96-well multi-titre microplates.

Cell viability was determined with MTT assay. The cells were seeded and incubated in sterile 96-well microplates at a density 1 × 10⁴ cells/well at 37 °C for 24 h. Following incubation, the cells were subjected to the treatment as detailed in the cell culture and treatment section, and incubated for 24 h. Thereafter, the culture media and the treatment dilutions were replaced with freshly-

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prepared bovine-free DMEM, followed by addition of 50 mg % MTT and incubation for 4 h at 37 °C. Next, the formazan crystals were solubilized in 100 µL DMSO. Absorbance was measured at 540 nm in an ELISA reader. The absorbance values of the blank wells were subtracted from that of each sample, and the IC₅₀ was calculated. The assay was done in triplicate, and the mean and standard error was calculated with GraphPad/Prism. Chromatin changes were determined with DAPI staining. The pigment was prepared in the dark at a concentration 20 mg/mL in DW, homogenized in an ultrasonic water bath, and preserved via refrigeration. The HeLa cell line was incubated in a humidified ambient environment with 5 % CO₂ at 37 °C for 24 h. After adherence and cell morphology examination, the cells were exposed to the test compound and standard at different concentrations (20–200 µg/mL). After incubation for 24 h in 5 % CO₂ and humidity at 37 °C, the cells were fixed in 4 % paraformaldehyde for 15 min, and then washed with PBS. Then, DAPI dye was added to the cell cultures (control and treated), left for 5 min in the dark, and analyzed under an inverted fluorescence microscope for apoptosis [15].

Statistical analysis

All experiments were performed with four independent replications, and data are expressed as mean value ± standard deviation (mean ± SD). Statistical analysis was made with one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test (for MTT assay data), and non-linear regression (for analysis of IC₅₀). Differences were reported as significant at p < 0.05.

RESULTS

Extraction and isolation of compounds

The percentages of yields obtained from the methanolic CE and fractionation portions were calculated for the total amount of crude material used. Table 1 shows the highest yield, with the EIOAc fraction (3.12 %) compared to the n-hexane (1.56 %).

The presence of terpenoids in the CE and the fractions as detected using TLC and chemical analyses is shown in Table 2. The highest amount of terpenoids was seen in the n-hexane extract, although some terpenoids were also detected in the ethyl acetate fraction.

Table 2: Secondary metabolites in Cyperus rotundus crude extract

| Extract/fraction | Sterols | Terpenes | Peroxide |
|------------------|---------|----------|----------|
| Crude methanol extract | ++ | +++ | ++ |
| n-Hexane | +++ | +++ | - |
| Chloroform | - | - | - |
| Ethyl acetate | - | ++ | ++ |
| n-Butanol | - | - | - |
| Aqueous | - | - | - |

The CE, n-hexane and ethyl acetate fractions exhibited antimicrobial activities against S. aureus and C. albicans (Table 3, supplementary). n-Hexane sub-fractions 7 and 28 eluted on silica gel showed larger inhibition zones (3.6 and 6.0 mm, respectively) against C. albicans, while low activity was obtained against S. aureus (Table 5s). The eluted n-hexane sub-fractions (1-6/9-20/21-31 and 13-21) and ethyl acetate (13-18/19-21) retained the same activity (Table 6s).

A single deep-yellow spot (50 mg) with a sticky texture recovered via preparative-TLC (PTLC) (Rf = 0.6) showed antimicrobial activity. The purity of the sample was confirmed using HPLC (Figure 8s).

Spectroscopy and structural elucidation

Results from FT/IR spectroscopic analysis showed a broad band at 3600 cm⁻¹ (C-H stretching at 3030 cm⁻¹; absorbance at 2890 and 2840 cm⁻¹ [C-H stretching for aliphatic (-CH₂ and -CH₃]), and overtone at 2000–1700 cm⁻¹.

| Partitioning | Flash Col. Chrom. 1st elution | Flash Col. Chrom. 2nd elution |
|--------------|-------------------------------|-------------------------------|
| Fraction | Yield (g) | Yield (%) | Sub-fraction | Yield (g) | Yield (%) | Sub-fraction | Yield (g) |
| n-Hexane | 23.4 | 1.56 | n-hexane | 11.05 | n-hexane | 5.6 |
| Chloroform | 14.6 | 0.97 | Ethyl acetate | 15.9 | Ethyl acetate | 11.4 |
| Ethyl acetate | 46.8 | 3.12 | - | - | - | - |
| n-Butanol | 26.3 | 1.75 | - | - | - | - |
| Aqueous | 83.5 | 5.56 | - | - | - | - |
| CE | 195 | 13 | - | - | - | - |
Aromatic (C=O) stretching was seen at 1604 cm\(^{-1}\) for activated aromatic ring (substituted). The peak at 1495 cm\(^{-1}\) was for C=C stretching (aromatic), while the absorbance at 1190 cm\(^{-1}\) represented stretching for C-O (alcohol). The band at 1030 cm\(^{-1}\) represented C-H bending for -CH\(_2\) group, and the bending bands (out of plane) at 894, 812, and 728 cm\(^{-1}\) were for C-H bending for substituted aromatic compounds. A band at 850 cm\(^{-1}\) was for stretching for peroxy group (C-O-O-C) (Figure 1).

The proton NMR (400 MHz, methanol-\(\text{d}_6\)) \(\delta\) ppm results (Figures 2 and 3) showed the following chemical shifts: 1.31 (s, 12H), 1.56-1.73 (m, 4H), 2.01-2.09 (m, 1H), 2.37 (t, \(J = 7.34\) Hz, 2H), 2.68 (s, 2H), 3.47-3.60 (s, 2H), 3.64-3.69 (m, 1H), 3.80-3.89 (m, 2H), 4.09 (d, \(J = 6.24\) Hz, 1H), 4.15 (d, \(J = 4.52\) Hz, 1H), 4.35 (d, \(J = 5.38\) Hz, 1H), 5.32 (d, \(J = 6.72\) Hz, 1H), 6.67 (d, \(J = 8.44\) Hz, 1H), 7.06 (d, \(J = 8.40\) Hz, 1H).

The results of spectroscopic analysis strongly supported the presence of glucose, peroxy group, activated aromatic ring, and symmetric methyl groups, which are all characteristic features of the isolated compound (3,9-peroxysesquiterpene-15-O-glucoside).

Figure 1: Absorbance (cm\(^{-1}\)) versus %T for the isolated 3,9-peroxysesquiterpene-15-O-glucoside, as analyzed using FT-IR (Perkin Elmer; 400-4000 cm\(^{-1}\)).

Figure 2: Proton NMR (400 MHz, methanol-\(\text{d}_6\)) chemical shifts (ppm) and coupling constant (\(J\)) with integrations for the isolated 3,9-peroxysesquiterpene-15-O-glucoside analyzed by \(^1\)H NMR Bruker.

Antimicrobial activity (MIC and MBC)

The MIC for the isolated compound showed antibacterial activity against \(S.\) \textit{aureus} and \(C.\) \textit{albicans} at a concentration of 32 \(\mu\)g/mL (Figures 4A and 4C). No activity was recorded against \(E.\) \textit{coli} (Figure 4B). The MBC for the previous concentration showed no growth (bactericidal effect) at a concentration of 64 \(\mu\)g/mL for \(S.\) \textit{aureus}, while the MBC against \(C.\) \textit{albicans} was 32 \(\mu\)g/mL. Similar MIC results were obtained for CE at concentrations of 64 and 100 \(\mu\)g/mL against \(S.\) \textit{aureus} and \(C.\) \textit{albicans}, respectively. The standard errors were 0.3 and 0.299, respectively, while no activity was seen against \(E.\) \textit{coli}. The MBC for CE showed growth of \(S.\) \textit{aureus} at all inhibitory dilutions, but no growth (bactericidal) at concentrations of 64 and 100 \(\mu\)g/mL for \(C.\) \textit{albicans} (Figure 10s).
Figure 4: Antimicrobial effects of the isolated 3,9-peroxysesquiterpene-15-O-glucoside obtained from ethyl acetate fraction against growth of S. aureus (A), E. coli (B), and C. albicans (C), respectively

MTT and DAPI assay results

The cytotoxicity of the isolated 3,9-peroxysesquiterpene-15-O-glucoside on human endothelial (HeLa) cancer cell line was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The test compound showed dose-dependent cytotoxic activity (IC$_{50}$ = 88.32 µgm/mL; Figure 5) after exposure to increasing concentrations (20–200 µg/mL), and reduced cell viability (Figure 6). Chromatin pigmentation revealed fragmented nuclei and apoptosis (Figure 7). Structural elucidation and bioactivity assessment confirmed that the isolated compound was 3,9-peroxysesquiterpene-15-O-glucoside, with very high antibacterial, antifungal, and cytotoxic profiles.

DISCUSSION

The extraction of higher yields of sterols, terpenes and peroxides from CR rhizomes, relative to reports in other studies, may be attributed to longer extraction time, altered extraction technique, and the solvents used, as well as differences in the environmental growth conditions of the plants [7,16]. Terpenoids were eluted better by refluxing with n-hex solvent, while high yield of terpenoids was obtained from the dichloromethane fraction [17]. In this study, the detection of bioactive terpenoids in the EtOAc fraction suggests post-translational structural modification of these terpenes, resulting in reduction in their lipophilic properties. Results from FT/IR analysis confirmed the presence of multiple hydroxyl (O−H) groups bonded with water, ether (C−O) group for glucose, phenol, phenyl ring and aliphatic hydrocarbons (−CH$_2$ and −CH$_3$), while the band at 850 cm$^{-1}$ represented the presence of a peroxy group in the compound [19], based on the indicated absorption wavelengths.

The assignment of protons using $^1$H NMR (400 MHz, methanol) showed chemical shifts δ at 7.06 ppm d, 6.67 ppm d, the integration (1H). They were coupled and splitting each other ($J = 8.40$ and 8.44 Hz), and they belong to the aromatic ring attached to C-6 and C-14. The ring was substituted with −CH$_2$O-glucose, losing one proton. The phenolic oxygen at C-12 has an inductive effect, making for mesomerism, and shifting the absorption downfield [16,19]. The H
NMR analysis showed the presence of glucose protons in the region 5.32–3.60 ppm (11H). Both anomeric and C-2 carbons exerted de-shielding effects on the anomeric proton and shifted the chemical shift downfield [20].

The two protons attached to C6’ appeared in the region 3.80–3.89 ppm (2H), splitting each other and the adjacent hydroxyl proton (H-C-OH). The chemical shift for glucose protons appears in the same region of the diluent (methanol) and were overlapped. The H NMR analysis showed the presence of glucose molecules in the spectrum, since the chemical shifts were typical for glucose moiety [21].

The methoxy group (C15) attached to the aromatic ring with two protons (2H) showed chemical shifts at 2.68 ppm s, de-shielded by the adjacent oxygen. The assignment for C-2 and C-8 protons showed chemical shift at 2.37 ppm t (symmetric two protons, 2H). These protons were coupled (J = 7.34 Hz). They appeared in this region because they were adjacent to the aromatic ring. These protons were symmetric and overlapped. The presence of a triplet peak indicated splitting to adjacent C-1 and C-10 with two protons attached [16,19]. The C-1 and C-10 protons (4H) showed chemical shifts at 1.56–1.73 ppm m, symmetric protons (two each) attached to carbons C-1 and C-10, and splitting each other (multiplet), and protons attached to both C-2 and C-8 [16]. The protons of the four methyl groups (4, 5, 16, and 17) (12H) were symmetric and appeared in the region 1.31 ppm s. The adjacent carbons (C-3 and C-9) had no protons, and were therefore, singlet (Figure 3).

The phenolic hydroxyl proton (1H) attached to C-12 of the aromatic ring showed at 2.01–2.09 ppm. This proton was affected by electron density (ortho inductive effect, mesomerism) and it appeared in the upfield rather than downfield (less electronegative effect of the phenolic oxygen) [19].

The 13C NMR (400 MHz, DMSO) analysis confirmed the attachment of glucose and showed the following chemical shifts: δ 70.5 ppm CH2 (6’), δ 77.2 ppm CH (C5’), δ 70.5 ppm CH (4’), δ 78.4 ppm CH (C3’), δ 72.6 ppm CH (C2’), δ 91.0 ppm CH (C1’), which were for glucose. The chemical shift for C-6 and C-14 showed δ 114.84 ppm CH, which were shielded by the strong electronegative field of the peroxo group and the tautomer additive effect of the phenolic hydroxyl on the benzene ring [22]. The 13C NMR analysis also showed a chemical shift for both carbons (C-2 and C-8) at δ 50.96 or 49.54 ppm CH, since they were symmetric. This is consistent with the results obtained previously [16]. The chemical shift for C-4, C-5, C-16, and C-17 from 13C NMR analysis showed δ 24.9 or 22.59 or 22.48 ppm CH3 (symmetric groups) shielded by the peroxo group [22]. The presence of the C-9 and C-13 proton group in the region δ = 82.97, 84.13, or 81.84 ppm was confirmed from the FT/IR assay, absorption band at 850 cm⁻¹, and results of chemical reaction.

Mass spectrum analysis using a high-resolution LCMsMs/ABSCIEX for the isolated peroxy sesquiterpene glycoside (C29H36O9) (Figure 11s) showed ion fragments (m/z 163.3 and 179) for (glucose-OH). The results from mass spectrum analysis as well as fragments of m/z = 453.5 (M)+, C29H36O9, are shown in Table 4, and Figures 12s and 13s.

The isolated sticky, deep-yellow sesquiterpene showed structural variations when compared to the isolated peroxysesquiterpene (peroxycalamenene) from CR rhizome reported previously [16]. The latter showed higher molecular mass which is attributable to further enzymatic post-synthesis modification (probably environmentally enhanced), resulting in additional bi-methylation on C-9, also attached to the oxygen of the peroxide bridge (not to C-8) [23]. The presence of the peroxide group was also confirmed by chemical reaction (yellow-brown color) [12]. The C-12 of the aromatic ring was further modified by substituting the phenolic hydroxyl group. The methyl group at C-15 was glycosylated as well, a reaction usually catalyzed by glycosyltransferase. Structural elucidation of the isolated compound from EtOAc fraction confirmed the presence of a 3,9-peroxysesquiterpene-15-O-glucoside (the numbering system was made according to the biogenetic origin of the linked isoprene units). The present study is the first report on the isolation of this compound from CR rhizomes.

The antimicrobial activity of crude extract of CR and its partitions were evaluated using disc diffusion assay against S. aureus, E. coli and C. albicans grown on MHAP. The CE extract and EtOAc fraction showed antimicrobial activity against S. aureus. However, no activity was recorded against E. coli, which might be due to impermeability of the bacterial outer membrane layer to the isolated compounds, as well as concentration effects and antimicrobial specificity of the isolated compound. In addition, biosynthesis of the secondary metabolites inside the plant cells may have been affected by long drought seasons in the region, which eventually affected the bioactivity potential of the extract [24]. The lack of activity of the isolated 3,9-peroxysesquiterpene-15-O-glucoside against E.
coli might be associated with the higher hydrophilicity of the compound which resulted in its poor penetration through the bacterial envelope.

The anti-fungal effects of the ethanol and essential-oil extracts of CR against C. albicans cells were determined. The results were similar to those obtained previously [25]. The MIC and MBC values of CE were 3 folds higher, when compared to the results obtained by Parekh and Chanda [26].

Hella Cells were treated using the purified compound at a concentration of 88 µg/mL, which showed cytotoxic activity, and the treated cells were stained using DNA-binding dye DAPI, which showed condensed chromatin, fragmented nuclei, and formation of apoptotic bodies. These features, which were concentration-dependent, are characteristics of apoptosis. The cytotoxic effect of the test compound was manifested in reduction in numbers with cytoplasm shrinkage (viability) and nuclei pyknosis. It has been reported that sesquiterpene lactones showed cytotoxicity on various human cancer cells. The cytotoxicity of the structurally related 10,12-peroxyxalamenene has not been studied. This study has demonstrated promising dose-dependent cytotoxic and apoptosis-enhancing effects of the isolated 3,9-peroxysesquiterpeneprene-15-O-glucoside.

The need for new cytotoxic lead compounds in drug discovery encourages further investigations into the structure-activity relationship of 3,9-peroxysesquiterpeneprene-15-O-glucoside, and the molecular mechanism behind its toxicity, including transcriptional analysis of the genes involved in the apoptotic pathway.

CONCLUSION

A novel sesquiterpene glucoside has been successfully isolated from the crude extract of indigenous CR rhizomes using sequential chromatographic techniques, and its structure determined using spectrometric analysis. The compound exhibits promising antibacterial and antifungal properties against S. aureus and C. albicans, as well as cytotoxic activity against human endothelial HeLa cell lines.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Kawkab Y. Saour, and Shwan Rachid supervised and contributed to planning of this work. Mohammed N. Sabir performed the experiments, and S. R. verified the results. MN. S. drafted the manuscript and designed the figures. The results were discussed by all authors, who provided critical feedback and were equally involved in preparing the final manuscript.

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