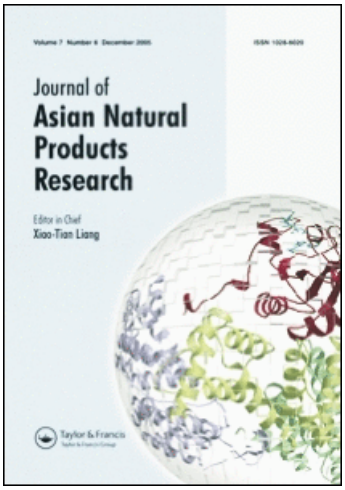

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Identification and quantification of the active component quercetin 3-*O*-rutinoside from *Barringtonia racemosa*, targets mitochondrial apoptotic pathway in acute lymphoblastic leukemia

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ORIGINAL ARTICLE

Identification and quantification of the active component quercetin 3-*O*-rutinoside from *Barringtonia racemosa*, targets mitochondrial apoptotic pathway in acute lymphoblastic leukemia

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Barringtonia racemosa has been used as a traditional medicine for the treatment of various diseases. The antitumor property of the seed extract of this plant in mice model promotes us to search for the active component present in the fruit extract. Quercetin 3-*O*-rutinoside (QOR) has been isolated from the fruits of this plant for the first time and quantified by HPLC method. The compound was identified by IR, mass, and NMR (1D, 2D) spectral data analysis. QOR showed dose- and time-dependent anti-proliferative activity in several leukemic cell lines with negligible effect on normal human peripheral blood mononuclear cell (PBMC). A representative T-lineage acute lymphoblastic leukemia cell line (MOLT-3) showed phosphatidyl serine externalization and DNA fragmentation, indicating QOR-induced programed cell death. We established that QOR-induced apoptosis occurred preferentially on accumulation of cells in the sub-G_0 phase and genomic DNA fragmentation through the activation of mitochondria-dependent caspase cascade for the first time in T-lineage ALL cell line.

Keywords: *Barringtonia racemosa*; Lecythidaceae; quercetin 3-*O*-rutinoside; acute lymphoblastic leukemia; mitochondrial apoptosis

1. Introduction

Barringtonia racemosa belongs to Lecythidaceae family spread in Bangladesh, Sri Lanka, and west coast of India [1]. According to ethnomedical survey, the various parts of this plant are traditionally used as a folk medicine to treat rat/snake bite, rat poisoning, gastric ulcers, itch, piles, and typhoid fever [1–6]. The bark of the plant showed antinociceptive properties and extensive antifungal activity [5]. Fruits are used in certain far-flung villages of Kerala, southern part of India, to treat cancer-like diseases [7]. The seeds of the plant displayed cytotoxicity against HeLa cell line [2] and Dalton's lymphoma ascetic cells [6].

Based on the existing information, *B. racemosa* should be considered as a potential medicinal plant. However, there is no report on the active component present in the fruit extract. Accordingly, we were interested to search for the active component from the bioactive-guided fractionations. Our search yielded quercetin 3-*O*-rutinoside (QOR) as the active component, which is isolated for the first time from the fruits of

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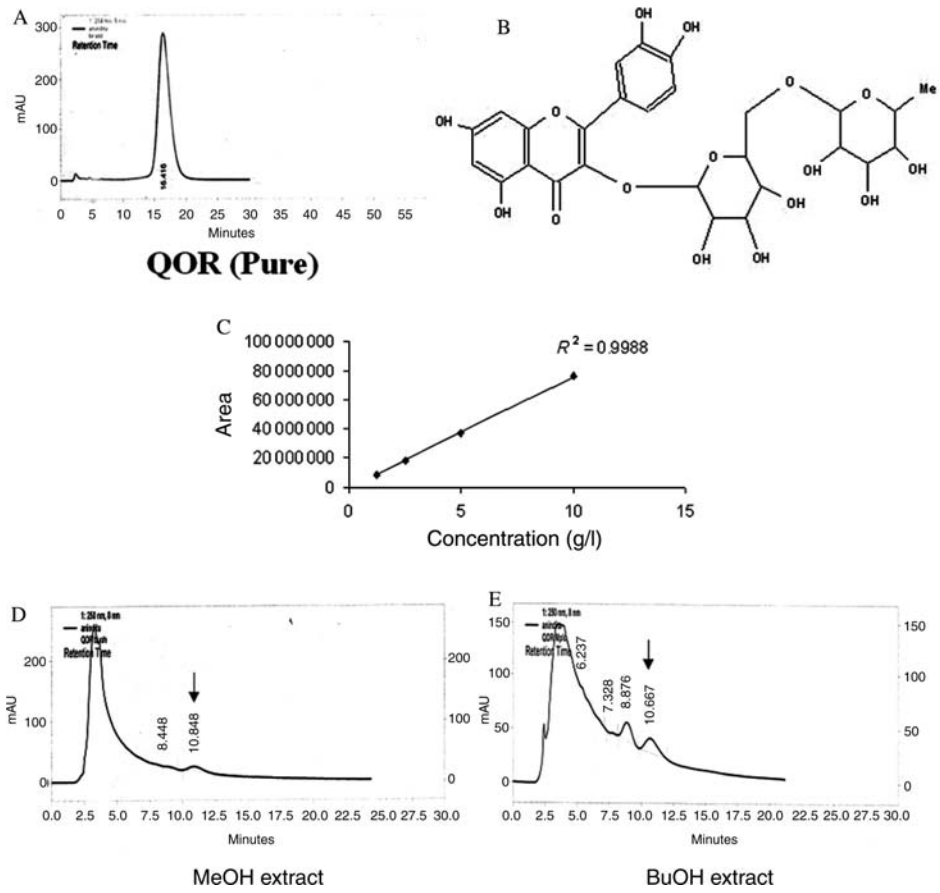

Figure 1. Identification and HPLC peak profile of QOR. (A) HPLC profile of purified QOR. (B) Struture of isolated QOR. (C) Calibration curve of standard QOR using HPLC. (D) HPLC chromatogram for methanolic extract and butanolic extract. Arrow indicates the peak of QOR.

this plant and analytically quantified by HPLC. QOR is identified through its physical and spectral data. The structure was elucidated mainly by NMR analysis, including 1D and 2D NMR (^{1}H–^{1}H COSY, TOCSY, NOESY, HSQC, and HMBC), IR, and mass spectrometry (Figure 1B).

Quercetin is the structurally related flavonoid with QOR, which has been demonstrated to work as a potential antitumor drug in several cancer types [8–12]. Previous reports indicated that QOR has been isolated from other plant sources like *Bauhinia monandra* and *Ilex hainanensis* Merr. [13] and showed cytotoxicity against lung carcinoma [14] and pancreatic carcinoma [15]. Chen *et al.* [16] showed antiproliferative effect on promyelocytic leukemia (HL-60) cells by using a mixture of three compounds containing QOR. But there is no report about the active component QOR present in the fruit extract of *B. recemosa* and its *in vitro* study on cancer cell line, especially T-lineage acute lymphoblastic leukemia (T-ALL).

In the present study, we have investigated the *in vitro* induction of cell death by the extract of *B. racemosa* and its active component QOR in T (JURKAT and MOLT-3) and B (REH) lineage of ALL and chronic myelogenous leukemic (K562) cell lines. Our results indicated

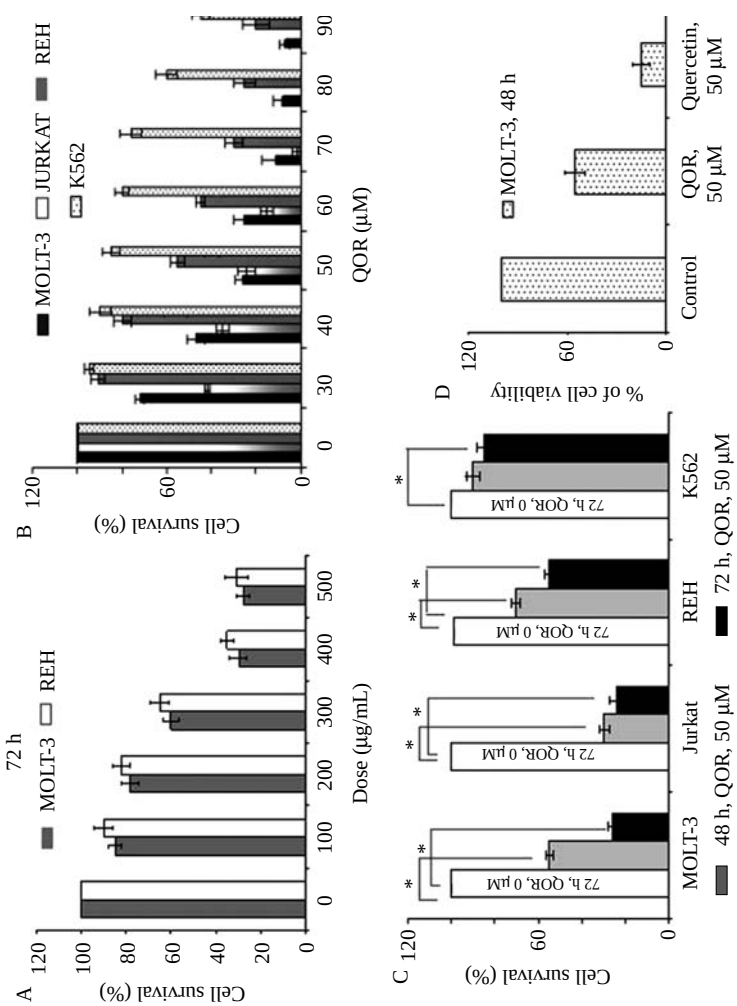

Figure 2. Cytotoxic effect of QOR in various cancerous cell lines. (A) Cytotoxic effect of the methanolic extract of *B. racemosa* on MOLT-3 and REH. (B) QOR-induced dose-dependent anti-proliferative activity in different cancer cell lines after 72 h of incubation as detected by MTT assay. (C) Time-dependent anti-proliferative activity in different cancer cell lines after treatment with QOR at a fixed dose of 50 μm by MTT assay. (D) Comparison of QOR with quercetin used as positive control. *P-value < 0.05 by Student t-test in Microsoft Excel is considered significant.

that QOR could induce anti-proliferative effect on the cell lines of T- and B-ALL. We have also demonstrated that QOR induced apoptosis preferentially in the leukemic cells and found to be associated with (1) mitochondria-mediated death signaling pathway, (2) activation of caspase cascade, and (3) genomic DNA fragmentation and subsequently accumulation of cells in the sub-G_0 phase.

2. Results and discussion

In the process of searching for antitumor-active component from Indian medicinal plants, we have selected the whole fruit of *B. racemosa* as our plant source. Initially, two representative leukemic cell lines (MOLT-3 and REH) were exposed to the methanolic extract (100–500 μg/ml) of the fruit of *B. racemosa*. We found that 100–200 μg/ml doses were very potent to inhibit those cancer cell proliferations up to 15–20% after 72 h of incubation (Figure 2A). To identify the main active component, the methanolic extract (503 g) of the fruit was suspended in H_2O and extracted with EtOAc to remove less polar compounds. Subsequently, three fractions (H_2O, EtOAc, and *n*-BuOH) of the methanolic extract were tested for cytotoxicity against these two leukemic cell lines, and the maximum activity was observed in the *n*-BuOH fraction. This fraction (4.2 g) was eluted from *n*-BuOH by the Diaion HP-20 column chromatography method in MeOH:H_2O (3:1) and showed maximum activity toward these cells. Then the eluted fraction in MeOH:H_2O (3:1) was subjected to MPLC and further eluted with MeOH:1% aqueous AcOH (2:3). Out of three MPLC eluted fractions, the second fraction showed an almost clear TLC spot and very effectively inhibited the leukemic cell proliferation. For further purification, fraction 2 was subjected to HPLC and eluted with MeOH:1% aqueous AcOH (2:3), and a most active purified compound was isolated and identified as QOR by spectral data analysis. The chromatogram obtained for MeOH extract, BuOH extract, and purified QOR (Figure 1A,D) showed a separate distinct peak for QOR. The calibration curve (Figure 1C) was prepared with standard QOR and was found to be linear ($r^2 = 0.998$) in the concentration range used. The concentration of QOR (Table 1) in MeOH extract was 1.2 ± 0.50 mg/g, and in BuOH fraction 12.69 ± 3.20 mg/g. The isolated QOR was found to be almost 100% pure. To the best of our knowledge, this is the first report on the existence of QOR in *B. racemosa* fruit and its activity toward T-ALL cell line.

To identify the anti-proliferative effect of QOR, T-ALL (MOLT-3, JURKAT), B-ALL (REH), and CML (K562) cell lines were exposed in 0–90 μM QOR for 48–72 h. The cell viability was checked by trypan blue exclusion assay, radioactive thymidine uptake assay, and the MTT assay. QOR showed anti-proliferative effect on leukemic cells in a dose- (Figure 2B) and time-dependent (Figure 2C) manner. The IC_{50} values varied between 40 and 55 μM after 72 h of incubation in MOLT-3, JURKAT, and REH (Table 2). In the

Table 1. Concentration of QOR determined in MeOH extract, *n*-BuOH, and purity of isolated QOR from *B. racemosa* fruit extract.

Sample	Concentration of QOR (mg/g or mg/ml)
Methanolic extract	1.2 ± 0.50 mg/g of MeOH extract
n-BuOH fraction	12.69 ± 3.10 mg/g of *n*-BuOH fraction
Isolated QOR	10.0 ± 0.01 mg/ml

Note: Mean ± standard deviation; $n = 3$.

Table 2. IC_{50} values of QOR in different cell lines and PBMC isolated from healthy individuals.

Name of cells and cell lines	IC_{50} at 72 h (μM) by three different experiments		
	Tryphan blue exclusion assay	MTT assay	[^{3}H]-Thymidine uptake assay
JURKAT	30 ± 4.00	25 ± 3.60	27 ± 3.20
MOLT 3	42 ± 3.00	39 ± 2.70	37 ± 4.00
REH	54 ± 5.00	55 ± 1.90	52 ± 2.50
K562	90 ± 5.00	86 ± 6.70	82 ± 2.10
PBMC	–	146 ± 5.00	–

viability assay, quercetin was used as a positive control because of the structural similarity with QOR (Figure 2D) [17]. However, interestingly, the IC_{50} value of K562 after 72 h was very high, around 90 μM (Table 2). This result indicated that QOR was not so active toward K562 cell line, as 90 μM QOR induced only 55% cell death in the case of K562, but at the same dose other cells were almost 70–80% dead. In parallel, normal PBMC was exposed with similar doses of QOR for 48 h under identical conditions. No toxic effect was observed even up to 80 μM dose, whereas the IC_{50} values for different cancer cell lines were around 40–55 μM.

Morphological study by phase contrast microscopy also recognized the changes in shape and size of QOR (50 μM) exposed MOLT-3 for 24 h (Figure 3A) suggesting that the QOR induced cell growth inhibition and death.

Thereafter, we wanted to explore whether the pattern of QOR-mediated cell growth inhibition is apoptosis or necrosis. Surface phosphatidyl serine (PS) externalization in cell surface is a hallmark of apoptosis [18,19]. Early apoptotic cells are only PS exposed, whereas late apoptotic cells loose their membrane integrity and stained with both PS-binding agent annexin V and DNA-binding dye propidium iodide (PI). The structurally related well-characterized flavonoid molecule, quercetin, is known to induce apoptosis in different kinds of cancer [20]. Accordingly, quercetin was used as the positive control to induce apoptosis (Figure 3B). Our finding established that QOR treatment triggered the surface PS externalization in MOLT-3 and early apoptotic cells (10.21%) were formed around 75 μM of QOR after 24 h of treatment (Figure 3C). Substantial proportions of both annexin V–PI positive late apoptotic cells (20.26%) were produced after 24 h in the presence of 100 μM QOR. Another hallmark of the apoptosis is the genomic DNA fragmentation [21,22]. QOR (75–100 μM) induced a considerable amount of DNA fragmentation in MOLT-3, as detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay after 24 h of exposure (Figure 3D), thus establishing the apoptosis.

In the cell cycle progression, apoptotic cells are accumulated in sub-G_0 phase in cell cycle analysis. This is also a concrete proof of apoptosis. QOR (75–100 μM) accumulated almost 15–20% of MOLT-3 cells in sub-G_0 phase in the cell cycle after 24 h of induction (Figure 4C). This signified that QOR mediated MOLT-3 apoptosis *in vitro*. So, PS externalization on cell surface, DNA fragmentation, and finally sub-G_0 phase accumulation of cells in cell cycle progression established the QOR-mediated apoptosis in MOLT-3.

We further questioned the pathway of QOR-mediated apoptosis in MOLT-3 and its dependency on mitochondria-mediated death cascade [21,23]. Mitochondrial

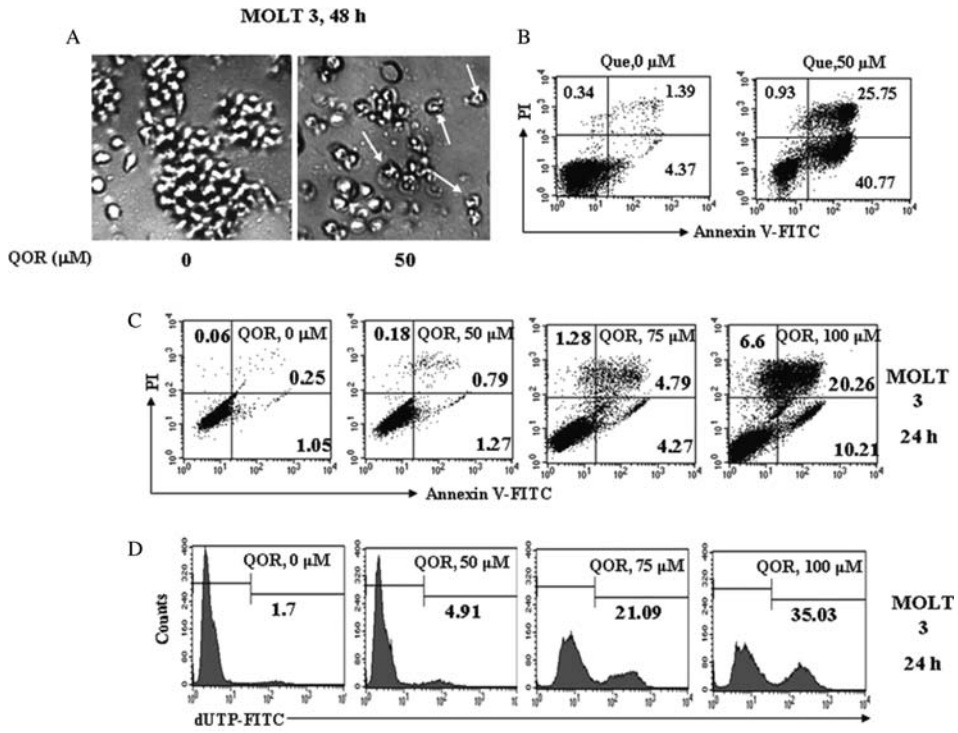

Figure 3. QOR induces apoptosis in MOLT-3 cell: (A) QOR-untreated and treated MOLT-3 after 48 h was observed under phase contrast microscopy. (B) Comparison of the pattern of cell death by quercetin as a positive control with QOR. (C) Flow cytometric analysis of annexin V-PI binding with untreated and QOR-treated cells with indicated dose after 24 h of incubation. Cell death occurred via primary and secondary apoptopic body formation. (D) QOR-mediated apoptosis was measured by TUNEL assay in MOLT-3 after 24 h of induction by flow cytometry.

membrane depolarization is one of the major events in this organelle-dependent apoptosis. The transmembrane potential drop of mitochondria after 24 h exposures with QOR was indicated with an increase of green fluorescence (FL-1) and decrease of red fluorescence (FL-2) of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) in a dose-dependent manner (Figure 4B). Here, in this experiment, we used 50 μM of quercetin as a positive control (Figure 4A).

Mitochondrial membrane depolarization event opened the field to roam in search of caspases [21,23]. We evaluated the surge of mito-dependent initiator caspase-9 and effector caspase-3. Both caspases were activated sequentially in a dose-dependent manner. The highest upsurge of caspase-9 at 40 μM was about 2.6-fold greater than that of the untreated control, whereas a 5.6-fold increase in caspase-3 was observed in the presence of 50 μM of QOR (Figure 4D). This observation further confirmed that the QOR activates mitochondrial-dependent apoptotic pathways in MOLT-3 cells.

In conclusion, QOR was isolated for the first time from *B. racemosa* fruit extract and induced mitochondrial apoptotic death cascade activation in T-lineage ALL cell line MOLT-3. Although, in the case of annexin V–PI binding both positivity occurred almost simultaneously, so from here we can hypothesize that except the

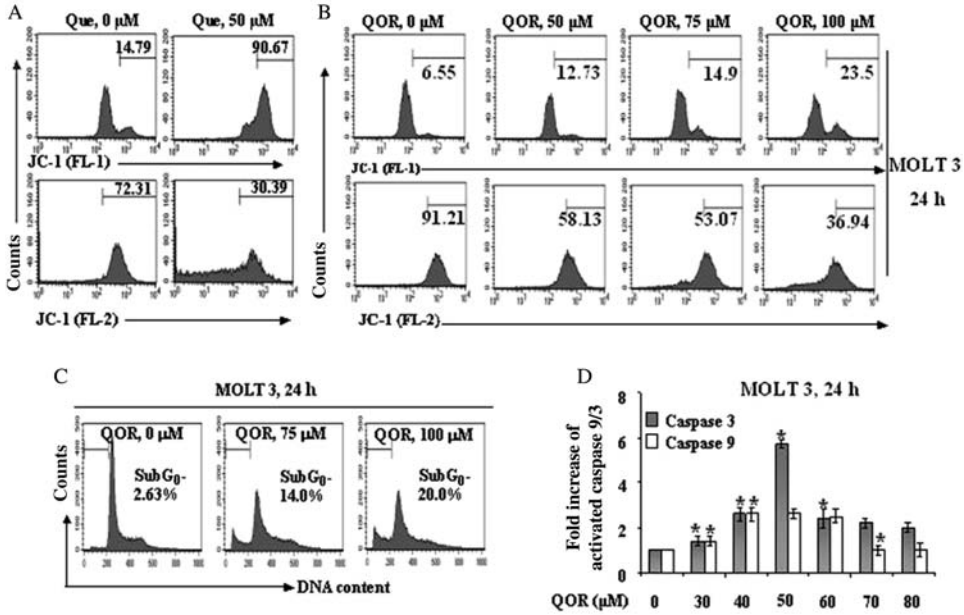

Figure 4. QOR-induced mitochondrial membrane depolarization and caspases-mediated apoptosis. (A) Quercetin-treated MOLT-3 was stained with JC-1 and compared with QOR-treated MOLT-3 after 24 h of incubation. (B) QOR-treated MOLT-3 after 24 h of incubation was stained with JC-1 and the transmembrane potential drop of mitochondria indicated by an increase of green fluorescence (FL-1) and decrease of red fluorescence (FL-2) of JC-1 in a dose-dependent manner by flow cytometry. (C) MOLT-3 cells were exposed to QOR in indicating dose for 24 h. Gates were set to assess the percentage of dead cells ($< 2n$ DNA). Bar denotes boundaries of sub-G_0 phase. (D) QOR-treated MOLT three cells at indicated dose for 24 h as well as for cells pretreated with pan caspase inhibitor Z-VAD-fmk (100 μm) was analyzed by flurometric method using fluorescent-labeled substrates according to the manufacturer's protocol. Caspases activation was expressed in terms of fold increase compared with untreated cells. The data shown are from a representative experiment performed three times with comparable results. The values reflect the mean ± SD of triplicate determination. $*P$-value < 0.05 considered as a significant value.

apoptotic cell death QOR might induce some other death machinery.

3. Method and materials

3.1 General experimental procedures

IR spectra were taken on a JASCO-FT-IR-model 410 spectrometer; ESI mass spectra (in both positive and negative modes) were analyzed in Q-TOF-Micromass Spectrometer. NMR spectra were recorded using Bruker Advance (600 MHz) in DMSO–d_6. TLC was carried out on silica gel 60 F254 (Merck, Darmstadt, Germany) plates using BuOH:AcOH:H_2O (9:5:7) as developing solvent and the spots were visualized by spraying with Liebermann–Burchard reagent followed by heating at 120°C. For HPLC, X-Terra preparative reverse phase C18 column (10 μm, 19 × 300 mm) was used [MeOH:1% aqueous AcOH (2:3), flow 23 ml/min, λ 254 nm]. Flow cytometry analysis was done by FACSCalibur Flow Cytometer Becton Dickinson (BD) and analyzed with the CellQuest Pro software.

3.2 Chemicals

Fetal bovine serum (FBS) was purchased from GIBCO-Invitrogen (Christchurch, New Zealand). PI and JC-1 were from

Molecular Probes – Invitrogen (Christchurch, New Zealand). Fluorescein isothiocyanate (FITC)–annexin V, fluorometric kits of caspases-3 and -9 were obtained from BD Biosciences (San Jose, CA, USA). MeOH, *n*-BuOH, EtOAC, AcOH, and HPLC-graded solvents were purchased from Spetrochem (Mumbai, India). Positive control quercetin (98% HPLC pure) was purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals unless otherwise mentioned were purchased from Sigma-Aldrich.

3.3 Plant materials

Fresh fruits of *B. racemosa* were collected (October 2007) from its natural habitat in the forest of Western Ghats region, Kerala, by United Chemicals and Allied Products, Kolkata, India. Dr N.D Paria, Department of Botany, University of Calcutta identified the plant material. A voucher specimen (IICB/Med-Chem-0211) has been deposited to the Department of Medicinal Chemistry, Indian Institute of Chemical Biology.

3.4 Isolation and purification

The dried fruits of *B. racemosa* were extracted with methanol at room temperature. The filtrate was concentrated to yield a methanol extract. The methanolic extract of the fruit was suspended in H_2O and extracted with EtOAc to remove less polar compounds. It was then extracted with *n*-BuOH. A portion of BuOH part was subjected to Diaion HP-20 column chromatography eluting with increasing concentration of MeOH in H_2O (1:1 → 100) to give three fractions. The fraction eluted with MeOH:H_2O (3:1) was subjected to MPLC eluting with MeOH:1% aqueous CH_3COOH (2:3), and we got three fractions (a–c) among which fraction b showed an almost clear TLC spot. For further purification, fraction b was subjected to HPLC eluting with MeOH-1% aqueous CH_3COOH (2:3), and ultimately we got a compound that gave a clear TLC spot. The HPLC chromatogram for MeOH extract, BuOH extract, and Isolated QOR are shown in Figure 1(A,D).

3.5 Identification of the compound

The QOR compound was obtained as a brownish crystalline solid; mp 212–214°C. It gave a dark-green color with ferric chloride reaction. Molecular formula $C_{27}H_{30}O_{16}$; Q-TOF-MS *m/z* 632.82 $[M + Na]^+$; by NMR (including 1D and 2D) and IR spectral data analysis, we identified the compound as QOR [13,24].

3.6 Quantification of QOR by HPLC

Precisely weighed samples (methanolic extract, butanol fraction, and QOR) were dissolved in running solvent [H_2O: MeOH:AcOH = 11:9:1 (v/v)] in an ultrasonic bath and filtered in 0.45 μm filter. An aliquot of 20 μl of each sample was injected into the HPLC column (XTerra™ and C_{18} column 5 μm, 4.6 × 250 mm; detector UV: 254 nm; flow: 1 ml/min). The external standard calibration curve for QOR was prepared with calibration solution within the concentration range of 1.25–10 mg/ml. Each solution was prepared and injected three times and the curve was constructed (in Microsoft Office Excel 2007) using an average of area. The calculated concentration of QOR was expressed in terms of mean ± SD (mg/g or mg/ml).

3.7 Cell culture

The human leukemic cell lines MOLT-3, REH, JURKAT, and K562 were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in the Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% FBS and 1% antibiotic antimycotic mixture (medium A) and cultured at 37°C in a humidified incubator containing 5% CO_2. PBMCs were isolated from the blood

samples of healthy individuals by Ficoll density gradient centrifugation.

3.8 Cell viability assay

The QOR compound was dissolved in dimethyl sulfoxide (DMSO) and was added to the culture medium to give a final DMSO concentration of 0.3% (v/v). This concentration of DMSO had no significant effect on the growth of the cell line tested. The cells (1×10^4/well) were seeded in the presence of different concentrations (0–90 μM) of QOR in a 96-well tissue culture plate for 48–72 h. [^{3}H]-Thymidine (0.1 μCi) was added 14–16 h before harvest time. Radioactivity was measured using a liquid scintillation counter (Packard Bioscience, Meridan, CT, USA). The viability of treated and untreated cells was also checked by MTT [25] and tryphan blue exclusion assay as described elsewhere [26].

3.9 Detection of surface PS externalization by flow cytometry

Cells were incubated for 24 h in the presence of varying concentrations of QOR (0–100 μM). The annexin V–PI-binding assay was performed using the annexin V–FITC apoptosis detection kit (BD Biosciences) according to the data sheet instruction. Apoptotic cells stained by annexin V are in the lower right quadrant. Later stage apoptotic cells stained with both annexin V and PI are in the upper right quadrant. Data acquisition was done on a FACSCalibur Flow Cytometer (BD) and analyzed with the CellQuest Pro software. Quercetin (50 μM) was used as positive control of apoptosis.

3.10 DNA fragmentation assays by flow cytometry

DNA fragments of QOR-untreated and treated cells were measured by terminal deoxynucleotidyl transferase (TDT)-mediated FITC–deoxyuridine triphosphate (FITC–dUTP) nick-end labeling (TUNEL) assay (APO-DIRECT, Phoneix Flow Systems, Inc.). QOR-exposed cells were initially fixed with 1% *para*-formaldehyde, followed by fixation with 70% ethanol. The DNA was subjected to an *in situ* tailing reaction in which residues of FITC–dUTP were added catalytically to the 3′-OH sites of DNA strand breaks by TDT.

3.11 Flow cytometric detection of mitochondrial transmembrane potential ($\Delta\Psi m$)

MOLT-3 cells (1×10^6/mL) were cultured with either the pure medium or the medium containing different concentrations of QOR (0–100 μM) for 24 h. After the incubation period, cells were washed twice with phosphate buffered saline (PBS). Then the cells were loaded with 25 μM JC-1 and incubated in 37°C for 30 min. Flow cytometric analysis for JC-1 fluorescence was done by a FACS-Calibur Flow Cytometer to determine the mitochondrial membrane potential of cells. Quercetin (50 μM) served as a positive control.

3.12 Measurement of caspase cascade activation

The enzyme activities of caspases-3 and -9 were measured using a caspase fluorometric assay kit (BioVision, Lyon, France). Cells were treated in six-well plates at a density of 1×10^6 cells/well. After the exposure of the cells to the QOR for different time periods (0–24 h), the cells were washed three times with PBS, lysed with lysis buffer, and incubated in ice for 10 min. The protein concentration was estimated by Lowry's method [27] with a protein of known concentration. Cell lysates containing 40 μg of protein were incubated with caspase-3 fluorogenic

substrate Asp-Glu-Val-Asp-(7-amino-4-trifluoromethylcoumarin) (DEVD-AFC), and caspase-9 fluorogenic substrate Leu-Glu-His-Asp-(7-amino-4-trifluoromethylcoumarin) (LEHD-AFC), for 1 h in 37°C. Caspase activity was measured by fluorometric detection using a micro-plate counter (excitation at 400 nm, emission at 505 nm).

3.13 Statistical analysis

Each experiment was performed at least three times and in duplicate. The considerable difference was estimated by Student's *t*-test. A difference of P (*) value (<0.05) was considered as significant.

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Note

1. Both Dr Bikas C. Pal and Dr Chitra Mandal are joint senior authors.

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