SUPPLEMENTARY MATERIAL

Bioactive chemical constituents from *Curcuma caesia* Roxb. rhizomes and inhibitory effect of curcuzederone on the migration of triple-negative breast cancer cell line MDA-MB-231

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Abstract

Rhizomes of *Curcuma caesia* are traditionally used to treat cancer in India. The aim is to isolate chemical constituents from *C. caesia* rhizomes through bioassay-guided fractionation. The extract, hexanes and chloroform fractions showed effect on MCF-7 and MDA-MB-231 cells in cell viability assay. The chromatographic separation afforded germacrone (1), zerumbone (2), furenodienone (3), curzerenone (4), curcumeneol (5), zederone (6), curcumenone (7), dehydrocurdione (8) from hexanes fraction and curcuminal G (9), curcuzederone (10), (1S, 10S), (4S,5S)-germacrone-1(10), 4-diepoxide (11), wenyujinin B (12), alismoxide (13), aerugidiol (14), zedoarolide B (15), zedoalactone B (16), zedoarondiol (17), isoedoarondiol (18) from chloroform fraction. This is first report of compounds 2, 9-13, 15-18 from *C. caesia*. The study demonstrated compounds 1-4 and 10 are the bioactive compounds. The effect of curcuzederone (10) on MDA-MB-231 cell migration showed significant inhibition in scratch and Transwell migration assays. The results revealed that curcuzederone could be a promising drug to treat cancer.

Keywords: *Curcuma caesia* Roxb.; cell viability assay; bioactive chemical constituents; cell migration; curcuzederone; breast cancer cell lines.

Experimental section

General

Organic solvents (MeOH, chloroform, ethyl acetate, hexanes, ethanol, HPLC grade ACN, and MeOH) and silica gel 60 (0.040–0.063 mm) for open column chromatography were purchased from Merck, Germany. NMR solvents (CDCl₃ and CD3OD) were purchased from ARMAR Chemicals, Switzerland. TLC plates pre-coated with Si₆₀ F₂₅₄ were purchased from Merck, Germany. The chemical constituents were isolated and purified from active fractions by using Prominence Shimadzu
Prep HPLC (PDA detector, dual pumps, Lab solutions Software) obtained from Shimadzu, Kyoto, Japan and Thermo Scientific Hypersil Gold RP-C18 column (250 x 21.2 mm I.D, particle size: 5 µ) purchased from Thermo Scientific, USA. \(^1\)H-NMR, \(^{13}\)C-NMR, and 2D NMR spectra were obtained from an ultra-shield Bruker DPX 500 spectrometer. All NMR spectra were recorded by using the residual solvent peaks (CDCl\(_3\), \(\delta\) 7.26, s, in \(^1\)H NMR and \(\delta\) 77.0, t, in \(^{13}\)C NMR, CD\(_2\)OD, \(\delta\) 3.31, m, in \(^1\)H NMR and \(\delta\) 49.2, m, in \(^{13}\)C NMR) as internal references.

**Plant materials and extraction**

The fresh rhizomes of *C caesia* were collected from Panchagarh, Bangladesh. Authentication of the plant sample has been confirmed by the taxonomist (Ms. Hosne Ara) of the National Herbarium of Bangladesh under the reference number DACB Accession No. 37513. The rhizomes were cleaned, washed with water, cut into small pieces, and sun-dried. The dried rhizomes were powdered by using a grinder machine to obtain fine powder (350 gm). The dried powder (340 g) was macerated in methanol (1500 ml×72 h×3 times) at room temperature. The extracting solvent was passed through filter paper to discard unwanted substances and concentrated *in vacuo* by a rotary evaporator under controlled temperature below 40°C. A total of 30 g crude methanol extract was obtained, and the yield was determined to be 8.82 % of the dried powder.

**Separation of the methanol extract**

The crude methanol extract (29 g) was applied to a vacuum liquid chromatography (VLC) and eluted with hexanes, chloroform, ethyl acetate and methanol to obtain four fractions: hexanes fraction (2.6 g, 9.0 % of crude extract), chloroform fraction (10.5 g, 36.2 %), ethyl acetate fraction (8 g, 27.6 %) and methanol fraction (6 g, 20.7 %). The effect of VLC fractions on breast cancer cells were evaluated by using cell viability assay.

Based on the activity, the most potent fraction (hexanes fraction, 400 mg) were subjected to RP-Prep HPLC which afforded compound 1 (17 mg, 0.38 % of crude extract), compound 2 (15 mg, 0.30 %), compound 3 (60 mg, 1.97 %), compound 4 (14 mg, 0.31 %), compound 5 (22 mg, 0.49 %), compound 6 (38 mg, 1.22 %), compound 7 (20 mg, 0.45 %), and compound 8 (45 mg, 1.51 %). The identification of chemical constituents have been carried out by comparison spectral data with those in the literatures and compounds 1-8 have been identified as germacrone (1) (Yamazaki et al. 1998), zerumbone (2) (Matthes et al. 1980), furanodienone (3) (Makabe et al. 2006), curzerenone (4) (Brieskorn and Noble 1982), curcumeneol (5) (Firman et al. 1988), zederone (6) (Hossain et al. 2015), curcumeneone (7) (Shiobara et al. 1985) and dehydrocurdione (8) (Firman et al. 1988).

Germacrone (1). Obtained as colourless prism. \(^1\)H-NMR (CDCl\(_3\), 500 MHz), \(\delta\) 1.43 (3H, s, H-14), 1.62 (3H, s, H-15), 1.72 (3H, s, H-12), 1.77 (3H, s, H-13), 2.06-2.18 (4H, m, H-2, H-3), 2.85 (1H, dd, \(J = 11.0, H-6\)), 2.95 (2H, dd, \(J = 12.0, 4.4\) Hz, H-9, H-6), 3.41 (1H, d, \(J = 10.6\) Hz, H-9), 4.70 (1H, dd, \(J = 2.0\) and 11.2 Hz, H-5), 4.98 (1H, d, \(J = 11.2\) Hz, H-1); \(^{13}\)C-NMR (CDCl\(_3\), 500 MHz).
8), 137.3 (C-11), 135.04 (C-10), 132.7 (C-1), 129.5 (C-7), 126.7 (C-4), 125.4 (C-5), 55.9 (C-9), 38.1 (C-3), 29.3(C-6), 24.1(C-2), 22.4 (C-13), 19.9 (C-12), 16.7 (C-15), 15.6 (C-14).

Zerumbone (2): Obtained as colourless crystals. $^1$H NMR (CDCl$_3$, 500 MHz): δ 1.07 (3H, s, 14-H/15-H), 1.20 (3H, s, 15-H/14-H), 1.54 (3H, s, 12-H), 1.79 (3H, s, 13-H), 1.89 (1H, d, J= 13.5 Hz, 1-Ha), 2.19–2.46 (5H, m, 1-Hb, 4-H2, 5-H2), 5.25 (1H, br. d, J= 15.5, 4.0 Hz, 2-H), 5.86 (1H, d, J= 16.4 Hz, 10-H), 5.97 (1H, d, J= 16.4 Hz, 9-H), 6.01 (1H, br. d, J= 11.6 Hz, 6-H); $^{13}$C NMR (CDCl$_3$, 500 MHz) δ 11.8 (C-13), 15.2 (C-12), 22.4 (C-14), 24.4 (C-5), 29.4 (C-15), 37.9 (C-11), 39.4 (C-4), 42.4(C-1), 125.0 (C-2), 127.2 (C-9), 136.3 (C-3), 138.0 (C-7), 148.9 (C-6), 160.8 (C-10), 204.4 (C-8).

Furanodienone (3). Obtained as colourless prism. $^1$H-NMR (CDCl$_3$, 500 MHz), δ 1.29 (3H, s, H-15), 1.84-2.47 (4H, m, H-2, H-3), 1.98 (3H, s, H-14), 2.11 (3H, s, H-13), 3.68 (2H, br. d, J=3.6 Hz H-9), 5.16 (1H, dd, J=4.6, 11.6 Hz, H-1), 5.79 (1H, s, H-5), 7.06 (1H, s, H-12); $^{13}$C-NMR (CDCl$_3$, 500 MHz), δ 189.8 (C-6), 156.5 (C-8), 145.8 (C-4), 138.1 (C-12), 135.4 (C-10), 132.5 (C-4), 130.5 (C-1), 123.7 (C-11), 122.2 (C-7), 41.7 (C-3), 40.7 (C-9), 26.5 (C-2), 19.0 (C-14), 15.8 (C-15), 9.6 (C-13).

Curzerenone (4): Obtained as colourless crystals. $^1$H-NMR (CDCl$_3$, 500 MHz), δ 1.18 (3H, s, H-15), 1.83 (3H, s, H-14), 2.17 (3H, s, H-13), 2.77 (1H, d, J=17.7 Hz, H-9), 2.90 (1H, d, J = 17.7 Hz, H-9), 3.01 (1H, s, H-5), 4.74 (2H, s, H-3), 4.97 (2H, m, H-2), 5.80 (1H, dd, J = 10.7, 17.5 Hz, H-1), 7.08 (1H, s, H-12); $^{13}$C-NMR (CDCl$_3$, 500 MHz), δ 194.9 (C-6), 165.5 (C-8), 145.5 (C-1), 141.1 (C-4), 139.5 (C-12), 120.1 (C-7), 119.2 (C-11), 115.6 (C-3), 113.0 (C-2), 64.1 (C-5), 42.8 (C-10), 33.6 (C-9), 24.9 (C-14), 24.8 (C-15), 9.0 (C-13).

Curcumenol (5): Obtained as colourless solid. $^1$H-NMR (CDCl$_3$, 500 MHz), δ 1.02 (3H, d, J=6.1 Hz, H-14), 1.59 (3H, s, H-12), 1.66 (3H, s, H-13), 1.82 (3H, s, H-15), 1.89 (6H, m, H-2, 3, 4), 2.07 (1H, d, J=14.7Hz, H-1), 2.13, 2.64 (2H, d, J= 15.0 Hz, H-6), 5.76 (1H, bd.s, H-9); $^{13}$C-NMR (CDCl$_3$, 500 MHz), δ 139.2 (C-7), 137.3 (C-10), 125.6 (C-9), 122.3 (C-11), 101.5(C-8), 85.7 (C-5), 51.2 (C-1), 40.4 (C-4), 37.2 (C-6), 31.2 (C-3), 27.6 (C-2), 22.3 (C-12), 21.0 (C-15), 18.9 (C-13), 11.9 (C-14).

Zederone (6): Obtained as colourless crystals. $^1$H-NMR (CDCl$_3$, 500 MHz), δ 1.28 (1H, m, H-3), 1.33 (3H, s, H-14), 1.60 (3H, s, H-15), 2.11 (3H, s, H-13), 2.28, (1H, m, H-2), 2.30 (1H, m, H-3), 2.49 (1H, m, H-2), 2.52 (1H, m, H-2), 3.66-3.77 (2H, m, H-9), 3.81 (1H, s, H-5), 5.47 (1H, d, J = 11.8 Hz, H-1); 7.08 (1H, br.s,H-12); $^{13}$C-NMR (CDCl$_3$, 500 MHz), δ 192.2 (C-6), 157.1 (C-8), 138.1 (C-12), 131.2 (C-1), 131.1 (C-10), 123.3 (C-7), 122.2 (C-11), 66.6 (C-5), 64.0 (C-4), 41.9 (C-9), 38.0 (C-3), 24.7 (C-2), 15.7 (C-15), 15.2 (C-14), 10.3 (C-13).

Curcumenate (7): Obtained as colourless solid. $^1$H-NMR (CDCl$_3$, 500 MHz), δ 0.43 (1H, m, H-1), 0.66 (1H, q, J=3.6 Hz, H-5), 1.11 (3H, s, H-15), 1.59 (2H, m, H-2), 1.78 (3H, s, H-13), 2.08 (3H, s, H-12), 2.12 (3H, s, H-14), 2.46 (2H, t, J=7.3 Hz, H-3); 2.52 (2H, d, J=8.1 Hz, H-9), 2.8 (2H, bd. s, H-6) $^{13}$C-NMR (CDCl$_3$, 500 MHz), δ 208.9 (C-4), 201.8 (C-8), 147.5 (C-11), 128.07 (C-7), 48.9
Dehydrocurdione (8): Obtained as colourless crystals. $^1$H-NMR (CDCl$_3$, 500 MHz), $\delta$ 1.02 (3H, d, $J = 7.0$ Hz, H-14), 1.64 (3H, s, H-15), 1.74 (3H, s, H-13), 1.77 (3H, s, H-12), 2.05 (2H, m, H-3), 2.11 (2H, m, H-2), 2.41 (1H, m, H-4), 3.06 (1H, d, $J = 11.1$ Hz, H-9), 3.27 (2H, dd, $J = 16.4$ Hz, H-9), 3.19-3.33 (2H, dd, $J = 15.8$, 4.0 Hz, H-6), 5.15 (1H, bd, H-1); $^{13}$C-NMR (CDCl$_3$, 500 MHz), $\delta$ 211.0 (C-5), 207.1 (C-8), 137.0 (C-11), 132.9 (C-1), 129.8 (C-10), 129.3 (C-7), 56.9 (C-9), 46.5 (C-4), 43.4 (C-6), 34.1 (C-3), 26.3 (C-2), 22.1 (C-11), 18.4 (C-14), 16.2 (C-15).

The chloroform fraction (10 g) which also showed effect on the viability of breast cancer cells were applied to silica gel column chromatography and eluted with hexanes-ethyl acetate (9:1), hexanes-ethyl acetate (8:2) and hexanes-ethyl acetate (1:1) to obtain Fr.1 (1.2 g, 4.1 % of crude extract), Fr.2 (4 g, 13.8 %) and Fr.3 (3.8 g, 13.1 %). The three new fractions (Fr.1-3, 100 mg each) were subjected to RP-Prep HPLC which afforded compound 9 (14 mg, 0.58 % of crude extract), compound 10 (25 mg, 1.03 %), compound 11 (13 mg, 1.79 %), compound 12 (12 mg, 1.66 %), compound 13 (14 mg, 1.93 %), compound 14 (28 mg, 3.86 %), compound 15 (26 mg, 3.59 %), compound 16 (12 mg, 1.57 %), compound 17 (35 mg, 4.59 %) and compound 18 (28 mg, 3.67 %) along with compound 3 (15 mg), compound 6 (9 mg) and compound 8 (12 mg). By comparison the spectral data with the literatures, compounds 9-18 have been identified as curcuminol G (9) (Ma et al. 2009), curcuzederone (10) (Eun et al. 2010), (1S, 10S), (4S,5S)-germacr-1(10), 4-diepoxide (11) (Harimaya et al. 1991), wenyujinin B (12) (Yin et al. 2014), alismoxide (13) (Oshima et al. 1983), aerugidiol (14) (Masuda et al. 1991), zedoarolide B (15) (Matsuda et al. 2001), zedoalactone B (16) zedoarondiol (17) ((Takano et al. 1995) and isozedoarondiol (18) (Kuroyanagi et al. 1987).

Curcuminol G (9): Obtained as colourless amorphous solid. $^1$H-NMR (CDCl$_3$, 500 MHz), $\delta$ 1.03 (3H, s, H-14), 1.68 (1H, m, H-1), 1.83 (3H, s, H-13), 1.90 (3H, s, H-15), 2.02 (1H, m, H-2), 2.06 (1H, m, H-1), 2.21 (1H, m, H-2), 2.29 (1H, d, $J = 13.5$ Hz,H-5), 2.42 (1H, m, H-10), 2.90 (1H, d, $J = 13.3$ Hz, H-5), 3.27 (1H, d, $J = 15.6$ Hz, H-8), 3.55 (1H, d, $J = 15.6$ Hz, H-8), 4.85 (1H, d, $J = 10.8$ Hz, H-3); $^{13}$C-NMR (CDCl$_3$, 500 MHz), $\delta$ 209.5 (C-9), 172.2 (C-12), 154.5 (C-7), 133.9 (C-3), 130.5 (C-4), 130.0 (C-11), 106.9 (C-6), 49.80 (C-5), 47.9 (C-10), 40.3 (C-8), 36.1 (C-1), 27.3 (C-2), 18.5 (C-14), 16.7 (C-15), 9.3 (C-13).

Curcuzederone (10). Obtained as colourless solid. $^1$H-NMR (CDCl$_3$, 500 MHz), $\delta$ 1.13 (3H, s, H-15), 1.32 (3H, s, H-14), 1.47 (1H, m, H-3$\beta$), 1.52 (1H, m, H-2$\beta$), 2.17 (3H, s, H-13), 2.23 (1H, brd, $J = 13.0$ Hz, H-2a), 2.40 (1H, brd, $J = 14.4$ Hz, H-3a), 2.82 (1H, d, $J = 16.7$ Hz, H-9a), 2.93 (1H, brd, $J = 12.0$ Hz, H-1), 3.68 (1H, d, $J = 16.7$ Hz, H-9$\beta$), 3.77 (1H, s, H-5), 7.09 (1H, s, H-13); $^{13}$C-NMR (CDCl$_3$, 500 MHz), $\delta$ 189.9 (C-6), 156.2 (C-8), 138.5 (C-12), 123.4 (C-11), 122.6 (C-7), 69.1 (C-1), 63.7 (C-4), 63.3 (C-5), 58 (C-10), 39.6(C-9), 36.1(C-3), 23.8 (C-2), 16.8 (C-15), 15.3 (C-14), 10.6 (C-13).
(1S,10S), (4S,5S)-Germacrone-1(10),4-diepoxide (11): Obtained as colourless solid. \(^1\)H-NMR (CDCl\(_3\), 500 MHz), \(\delta\) 1.13 (3H, s, H-14), 1.43 (3H, s, H-15), 1.78 (3H, s, H-11/12), 1.85 (3H, s, H-11/12), 2.04 (1H, m, H-3), 2.20 (1H, m, H-2), 2.26 (1H, dd, \(J=11.20\), 3.7 Hz, H-6), 2.62 (1H, dd, \(J=2.3\), 5.0 Hz, H-9), 2.64 (1H, dd, \(J=2.0\), 5.3 Hz, H-6), 2.84 (1H, dd, \(J=1.8\), 14.0 Hz, H-5), 2.90 (1H, d, \(J=10.5\) Hz, H-1), 2.99 (1H, d, \(J=10.7\) Hz, H-9); \(^13\)C-NMR (CDCl\(_3\), 500 MHz), \(\delta\) 207.3 (C-8), 138.0 (C-11), 134.3 (C-7), 64.1 (C-5), 61.4 (C-1), 60.2 (C-4), 58.5 (C-10), 54.5 (C-9), 35.7 (C-3), 29.3 (C-6), 23.0 (C-2), 22.8 (C-12), 20.8 (C-13), 17.4 (C-15), 15.6 (C-14).

Wenyujinin B (12): Obtained as colourless amorphous solid. \(^1\)H-NMR (CDCl\(_3\), 500 MHz), \(\delta\) 1.02 (3H, d, \(J=6.8\), H-14), 1.19 (3H, s, H-15), 1.24 (1H, m, H-3b), 1.38 (1H, m, H-2\(\beta\)), 1.88 (1H, m, H-3a), 1.92 (3H, s, H-13), 1.94 (1H, m, H-2\(\alpha\)), 2.05 (1H, d, \(J=14.0\) Hz, H-6\(\beta\)), 2.11 (3H, s, H-12), 2.12 (1H, m, H-1), 2.15 (1H, m, H-4), 2.43 (1H, d, \(J=16.0\) Hz, H-9\(\alpha\)), 2.76 (1H, d, \(J=14.1\) Hz, H-6\(\alpha\)), 3.19 (1H, d, \(J=16.2\) Hz, H-9\(\beta\)); \(^13\)C-NMR (CDCl\(_3\), 500 MHz), \(\delta\) 202.8 (C-8), 149.7 (C-11), 129.2 (C-7), 81.5 (C-5), 74.3 (C-10), 58.6 (C-1), 50.5 (C-9), 45.0 (C-4), 32.5 (C-15), 28.7 (C-6), 27.5 (C-3), 24.0 (C-12), 23.9 (C-13), 23.5 (C-2), 13.1 (C-14).

Alismoxide (13): Obtained as colourless amorphous solid. \(^1\)H-NMR (CDCl\(_3\), 500 MHz), \(\delta\) 0.97 (6H, dd, \(J=4.7\), 6.8 Hz, H-12 and H-13), 1.20, 1.26 (each 3H, s, H-14 and H-15), 5.48 (1H, d, \(J=3.0\) Hz, H-6); \(^13\)C-NMR (CDCl\(_3\), 500 MHz), \(\delta\) 149.7 (C-7), 121.3 (C-6), 80.3 (C-4), 75.4 (C-10), 50.6 (C-1), 50.3 (C-5), 42.6 (C-3), 40.4 (C-9), 37.3 (C-11), 25.1 (C-2), 22.5 (C-14/15), 21.5 (C-8), 21.4 (C-14/15), 21.3 (C-12/13), 21.2 (C-12/13).

Aerugidiol (14). Obtained as colourless solid. \(^1\)H-NMR (CDCl\(_3\), 500 MHz), \(\delta\) 1.41 (3H, s, H-14), 1.78 (1H, m, H-3), 1.82 (1H, dd, \(J=5.2\), 1.64 H-6), 1.85 (3H, s, H-13), 1.90 (1H, m, H-2), 1.98 (3H, d, \(J=5.6\) Hz, H-15), 2.02 (1H, m, H-3), 2.08 (1H, d, \(J=9.0\) Hz, H-5), 2.12 (1H, m, H-2), 2.15 (3H, s, H-12), 2.22 (1H, d, \(J=13.4\) Hz, H-3), 2.62 (1H, d, \(J=14.4\) Hz, H-9); \(^13\)C-NMR (CDCl\(_3\), 500 MHz), \(\delta\) 194.8 (C-8), 151.6 (C-10), 144.0 (C-11), 133.4 (C-7), 128.4 (C-9), 87.0 (C-1), 83.8 (C-4), 61.3 (C-5), 37.6 (C-2), 37.4 (C-3), 27.6 (C-6), 24.5 (C-14), 23.2 (C-12), 22.5 (C-13), 22.4 (C-15).

Zedoarolide B (15). Obtained as colourless solid. \(^1\)H-NMR (CD\(_2\)OD, 500 MHz), \(\delta\) 1.20 (3H, s, H-14), 1.34 (3H, s, H-15), 1.79 (3H, s, H-13), 1.58 (1H, m, H-2\(\alpha\)), 1.72 (1H, m, H-3\(\beta\)), 1.82 (1H, m, H-2\(\beta\)), 1.84 (1H, m, H-3\(\alpha\)), 2.01 (2H, dd, \(J=6.8\), 12.7 Hz, H-6\(\beta\), H-5), 2.22 (1H, d, \(J=15.4\) Hz, H-9\(\beta\)), 2.33 (1H, d, \(J=15.4\) Hz, H-9\(\alpha\)), 2.61 (1H, dd, \(J=2.9\), 15.4 Hz, H-6\(\alpha\)), 2.66 (1H, m, H-1); \(^13\)C-NMR (CD\(_2\)OD, 500 MHz), \(\delta\) 175.4 (C-12), 162.2 (C-7), 124.2 (C-11), 107.4 (C-8), 82.5 (C-4), 73.6 (C-10), 53.1 (C-1), 52.5 (C-5), 44.0 (C-9), 38.1 (C-3), 31.8 (C-14), 25.8 (C-2), 25.0 (C-15), 24.9 (C-6), 8.0 (C-13).

Zedoalactone B (16). Obtained as colourless solid. \(^1\)H-NMR (CDCl\(_3\), 500 MHz), \(\delta\) 1.21 (3H, s, H-15), 1.40 (3H, s, H-14), 1.48 (1H, m, H-2\(\alpha\)), 1.76 (2H, m, H-3), 1.78 (1H, dd, \(J=8.7\), 15.0 Hz, H-6\(\alpha\)), 1.80 (3H, d, \(J=1.8\) Hz, H-13), 1.84 (1H, m, H-2\(\beta\)), 1.98 (1H, m, H-5), 2.07 (1H, dd, \(J=16.7\), 2.0 Hz, H-9\(\alpha\)), 2.29 (1H, dd, \(J=7.0\), 16.0 Hz, H-9\(\beta\)), 2.70 (1H, ddd, \(J=3.4\), 7.4, 12.3 Hz, H-6\(\beta\)), 2.70 (1H, ddd,
Briefly, MDA MB cells were seeded into a 96-well plate at a density of 10 x10^4 and 4x10^4 cells/well, respectively and incubated for 24 h at 37°C. After incubation, the cells were treated with the crude extract, VLC fractions, pure compounds and 0.1 % DMSO as control and incubated for 48 h at 37°C. Then MTT solution (20 µl) was added into each well, incubated for 3 h and the absorbance was measured by using plate reader at a wavelength of 570 nm (Reference wavelength: 630 nm). Percentage of the cell viability of extract, fractions and pure compounds treated MDA-MB-231 and MCF-7 cells were calculated from the cell viability of the control. Scratch assay

The inhibitory effect of curcuzederone (10) on the migration of TNBC cell line MDA-MB-231 was evaluated by using scratch assay. Briefly, MDA-MB-231 cells were seeded into a 6-wells plate and incubated at 37 °C until reached 80 % confluence. Following incubation, a 100 µl micropipette tip was used to make a straight scratch in the middle of each well and washed the cellular debris with 200 µl PBS. The cells were treated with curcuzederone (10) and 0.1 % DMSO as control and incubated for

\[ J = 3.4, 7.4, 12.3 \text{ Hz, H-1}, 4.91 (1H, ddd, J=2.0, 2.7, 4.7 \text{ Hz, H-8}); ^{13}\text{C-NMR (CDCl}_3, 500 \text{ MHz), } \delta 8.1 (C-13), 24.6 (C-2), 25.0 (C-6), 25.1 (C-14), 32.0 (C-15), 35.8 (C-9), 37.2 (C-3), 51.0 (C-5), 51.6 (C-1), 73.6(C-10), 80.8(C-8), 81.8 (C-4), 122.8 (C-11), 161.2 (C-7), 175.5 (C-12) \]

Zedoarondiol (17). Obtained as colourless solid. ^1H-NMR (CD_3OD, 500 MHz), δ 1.17 (3H, s, H-14), 1.19 (3H, s, H-15), 1.81 (3H, s, H-13), 1.91 (1H, s, H-12), 1.38 (1H, m, H-5), 1.64-1.78 (4H, m, H-2, H-3), 1.92-2.02 (2H, m, H-1, H-6), 2.58 (1H, d, J=12.8 Hz, H-9), 2.80 (1H, d, J=15.0 Hz, H-6), 2.94 (1H, d, J=12.6 Hz, H-9); ^13C-NMR (CD_3OD, 500 MHz), δ 203.3 (C-8), 142.3 (C-11), 134.7 (C-7), 80.0 (C-4), 72.8 (C-10), 59.8 (C-9), 55.9 (C-1), 51.9 (C-5), 39.7 (C-3), 28.5 (C-6), 22.9 (C-2), 22.6 (C-12), 22.2 (C-15), 21.9 (C-13), 20.5 (C-14).

Isozedoarondiol (18). Obtained as colourless solid. ^1H-NMR (CD_3OD, 500 MHz), δ 1.22 (3H, s, H-14), 1.42 (3H, s, H-15), 1.86 (3H, s, H-13/H-12), 2.01 (1H, s, H-12/13), 2.42 (1H, d, J=16.2 Hz, H-9β), 3.22 (1H, d, J=16.3 Hz, H-9α); ^13C-NMR (CD_3OD, 500 MHz), δ 203.1 (C-8), 144.1 (C-11), 133.9 (C-7), 82.5 (C-4), 73.3 (C-10), 53.3 (C-1), 51.7 (C-5), 50.2 (C-9), 37.0 (C-6), 32.2 (C-15), 27.4 (C-3), 25.2 (C-2), 25.0 (C-14), 22.9 (C-13), 22.2 (C-12).

Cell Culture

Human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from ATCC, Rockville, MD, USA and DMEM supplemented with 10 % FBS was used to maintain the cells. The confluent MDA-MB-231 and MCF-7 cells were sub-cultured by using 0.25 % trypsin- EDTA and incubated at 37°C by maintaining humidified atmosphere with 5 % CO₂.

Cell viability assay

The effect of the crude extract, VLC fractions and pure compounds on breast cancer cells MDA-MB-231 and MCF-7 were investigated by using cell viability assay. Briefly, MDA MB-231 and MCF-7 cells were seeded into a 96-well plate at a density of 10 x10^4 and 4x10^4 cells/well, respectively and incubated for 24 h at 37°C. After incubation, the cells were treated with the crude extract, VLC fractions, pure compounds and 0.1 % DMSO as control and incubated for 48 h at 37°C. Then MTT solution (20 µl) was added into each well, incubated for 3 h and the absorbance was measured by using plate reader at a wavelength of 570 nm (Reference wavelength: 630 nm). Percentage of the cell viability of extract, fractions and pure compounds treated MDA-MB-231 and MCF-7 cells were calculated from the cell viability of the control.
24 h at 37 °C. The photographs of scratch were taken at 0 and 24 h by using Canon digital camera attached with a microscope at the magnification of 200 x. The percentage of wound closure of treatments and control was calculated by comparison the surface area of scratch at 24 h from the surface area of 0 h.

**Transwell migration assay**

The inhibitory effect of curcuzederone (10) on the migration of MDA-MB-231 cells was further evaluated by using Transwell migration assay. Briefly, MDA-MB-231 cells were suspended in DMEM and curcuzederone (10) at the doses of 50, 100, 150 µM and 0.1 % DMSO as control were added to the DMEM. After 30 min, 100 µl cells suspension (3 x 10⁵ cells/ml) were placed into upper chamber of each insert. The curcuzederone (10) at the doses of 50, 100, 150 µM and 0.1 % DMSO were also added in DMEM containing 10 % FBS (Complete DMEM). Then 500 µl complete DMEM containing treatments and control were placed to the lower chamber and incubated for 24 h at 37 °C. After incubation, the cells that remained on the upper side wall of the insert were removed by using cotton swabs. The cells that migrated on the lower side were fixed with 70 % ethanol and stained with 1 % crystal violet in 20 % ethanol. The light microscope was used to visualize the migrated cells at a magnification of 200 x and photographs from five fields of each insert were taken by using Canon digital camera. The number of migrated cells was counted and statistically analysed.

**Statistical analyses**

The experimental data are presented as the means ± SEM of three independent experiments. One-way ANOVA followed by Dunnett's multiple comparison tests was used to determine the significance. Statistical data were generated by using Microsoft Excel 2016 and SPSS (Version 23) software package for Windows (Chicago, IL, USA).

**References**


Table S1. IC₅₀ values of the crude extract, fractions and compounds 1-18 against breast cancer cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCF-7 (µg/ml)</th>
<th>IC₅₀ (µM)</th>
<th>MDA-MB-231 (µg/ml)</th>
<th>IC₅₀ (µM)</th>
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<td>MeOH extract</td>
<td>78.0±1.9</td>
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<td>Hexanes fraction</td>
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<td>Chloroform fraction</td>
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<td>Ethyl acetate fraction</td>
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<td>Methanol fraction</td>
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<td>Germacrone (1)</td>
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<td>Zerumbone (2)</td>
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<td>Furanodienone (3)</td>
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<td>Curzerenone (4)</td>
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<td>Curcuzederone (10)</td>
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<td>(1S,10S)(4S,5S)-germacrone-1(10),4-diepoxide (11)</td>
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<td>Tamoxifen</td>
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</table>

Results are mean±SEM of three independent experiments. NA: Not Active at the dose of 200 µg/ml for fractions and 400 µM for isolated compounds.
Figure S1. Chemical structures of compounds 1-18 from *C. caesia* Roxb. rhizomes.

Figure S2. Effect of curcuzederone (10) on MDA-MB-231 cell migration. (A) Photographic representation of MDA-MB-231 cell migration at the magnification of 200x after treated with control and compound for 24 h. (B) Percentage of wound closure. Results are mean±SEM of three independent experiments. *p <0.5, ***p <0.001, when compared to the control.
Figure S3. Inhibitory effect of curcuzederone (10) on MDA-MB-231 cell migration. (A) Photographic representation of MDA-MB-231 cell migration after treatment (B) Percentage of MDA-MB-231 cell migration treated with curcuzederone (10) at the doses of 50, 100 and 150 µM, when compared to control. Results are mean±SEM of three independent experiments. **p<0.001, when compared to the control.