

**CYTOTOXIC ACTIVITY OF RACEMOSAL AND DEMETHYLACEMOSAL FROM
BAUHINIA MALABARICA AGAINST HUMAN COLON CANCER COLO 205 CELLS****Suchada Jongrungruangchok^{1*}, Nalinee Pradubay², Thanapat Songsak³ and
Supakit Wongwiwatthanakut⁴**¹Department of Pharmaceutical chemistry, Faculty of Pharmacy, Rangsit University,
PathumThani 12000, Thailand²Department of Pharmacology, Faculty of Pharmacy, Rangsit University, PathumThani 12000, Thailand³Department of Pharmacognosy, Faculty of Pharmacy, Rangsit University, PathumThani 12000, Thailand⁴Department of Pharmaceutical Sciences, The Daniel K. Inouye College of Pharmacy,
University of Hawaii at Hilo, Hilo, HI

*Corresponding author: E-mail: suchada.j@rsu.ac.th

Abstract: *Bauhinia malabarica* Roxb.(*Caesalpiniaceae*) has been used in Thai traditional medicine for analgesic, antiinflammatory, antineoplastic, antidiabetic, antiulcers, antileprosy, antibronchitis, antiamoebic, antidysenteric, and hypocholesterolemic. Racemosal and demethylracemosal were isolated from the root of *B. malabarica* and were identified by NMR spectroscopy and mass spectroscopy. MTT assay showed that racemosal and demethylracemosal significantly exhibited cytotoxic activity against human colon cancer colo 205 cells exhibiting the IC₅₀ values of 6.16 and 6.45 μ M, respectively.

Keywords: *Bauhinia malabarica*, racemosal, demethylracemosal, ความเป็นพิษต่อเซลล์, colo 205 cells

บทคัดย่อ: ไม้ Bauhinia malabarica Roxb.(Caesalpiniaceae) ได้ใช้เป็นยาในการแพทย์แผนไทย ยาแก้ปวด ยาต้านการอักเสบ ยารักษาเบาหวาน ยารักษาแผลในกระเพาะอาหาร ยารักษาโรคเรื้อน ยารักษาหลอดลมอักเสบ ยารักษาโรคบิด ยาแก้บิด ยาลดระดับคอเลสเตอรอล สาร Racemosal และสาร demethylracemosal แยกได้จากรากของต้น Bauhinia ในการพิสูจน์เอกลักษณ์สาร โดยวิธี เอ็นเอ็มอาร์ สเปกโตรสโกปี และแมส สเปกโตรสโกปี การทดสอบความเป็นพิษต่อเซลล์มะเร็งลำไส้ colo 205 ด้วยวิธี MTT assay พบว่ามีค่า IC₅₀ เท่ากับ 6.16 และ 6.45 ไมโครโมลาร์ ตามลำดับ

คำสำคัญ: Bauhinia malabarica, racemosal, demethylracemosal, cytotoxic activity, colo 205 cells

INTRODUCTION

Natural products are one of the most significant sources as complementary and alternate therapy for treating and preventing of various human diseases, (Dahanukar, et al., 2000; Cowan, 1999). Phytochemical, pharmacological and allied approaches including various instrumental techniques are the fundamental knowledge for the usage of medicinal plants in preparation of such formulations (Panda et al., 2015). The use of plant extracts and phytochemicals have the advantage of being effective, simple, and safety with an emphasis on the preventive action of drugs. Consequently, phytopharmaceuticals have been increasingly utilized worldwide. The genus *Bauhinia* comprises about 300 species growing in most tropical area (Cechinel, 2009). *Bauhinia* trees typically reach a height of 6–12 m and their branches spread 3–6 m outwards. The leaves are green and broader than long. The lobed leaves usually are 10–15 cm across. The five-petaled flowers are 7.5–12.5 cm diameter, generally in shades of red, pink, purple, orange, or yellow, and are often fragrant. The tree begins flowering in late winter and often continues to flower into early summer. Depending on the species, *Bauhinia* flowers are usually pink, pale yellow or white, magenta, mauve, hues with bright crimson (Nirmal et al., 2011). Pods are flat, long about 17-35 x 1.5-2.5 cm. Seeds are flat, wide about 6-8 mm. Their leaves, flower buds, stem-bark and roots have been used in folk medicines of Africa, Asia, Middle and South America and have confirmed by the research *Bauhinia* species as remedies, such as analgesic, antiinflammatory, antineoplastic, antidiabetic, antiulcers, antileprosy, antibronchitis, antiamebic, antidyenteric, and hypocholesterolemic (Cechinel, 2000; Duarte-Almeida et al., 2004; Rajkapoor et al., 2006; Fuentes and Alarcón, 2006).

The crude extracts from stem of *B. guianensis* indicated notable anti-inflammatory and analgesic activities (Carvalho et al., 1999). Tetracyclic racemosol, lupeol, betulin, β -sitosterol, and tetracyclic 2, 2-dimethyl chroman have been isolated from the roots (Jain et al., 2002; Prabhakar et al., 1994; Anjaneyulu et al., 1986; Songarsa et al., 2005). Kittakoo et al (2000) reported that preracemosols A, B which are biogenetic precursors of the tetracyclic racemosol and demethylracemosol, the isolated from the roots of *B. malabarica* exhibited moderate antimalarial activity. The seed contains flavonoids, crude protein, and lipid (Mohan et al., 1994).

The methanol extract of *Bauhinia* leaves exhibited anti-oxidant activity attributed to the presence of cyclohexenone, lignans, and phenylethanoids derivatives (Sosa et al., 2002). Different parts of *B. purpurea* provided four *Bauhinia* statins as cancer cell growth inhibitors (Pettit et al., 2006). Treatment with the methanol extract of *B. racemosa* protects Wister albino rats against N-nitrosodiethylamine-induced hepatocarcinogenesis via suppressing nodule development and decreasing lipid peroxidation (Kumar et al., 2005). The alcohol extract of the stem bark of *B. variegata* showed a significant hepatoprotective activity against carbon tetrachloride intoxicated Sprague-Dawley rats (Bodakhe and Ram, 2007).

Hence the present work investigated the cytotoxic activity against human colon cancer cells (COLO 205) from the root *B. malabarica* Roxb. the bioactivity-guided fractionate on and chemical identification were carried out.

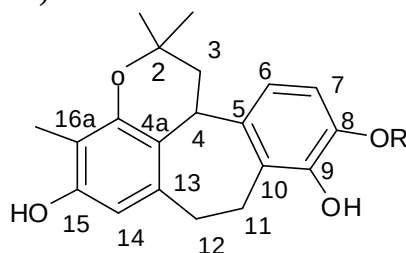
MATERIALS AND METHODS

Plant collection

The root of *B. malabarica* was collected from local region of Mahasarakham Province in November, 2014. The roots were identified by Dr. Thanapat Songsak by comparison with the authentic sample (voucher specimen no. BK32699).

Preparation of extract

The roots of *B. malabarica* (1268.7 g) were cleaned, dried at 45-50 °C and crushed to produce coarse powder for the extraction purpose. It was then macerated in dichloromethane (8 liters) successively. The extraction was carried out with CH₂Cl₂ solvent, by cold maceration process. The extracts were filtered through Whatmann filter paper to remove any impurities if present. The extracts were concentrated by vacuum distillation to reduce the volume. The eluate was evaporated under reduced pressure at 50 °C to obtain 1.35 g of CH₂Cl₂ extract. The CH₂Cl₂ layer was fractionized by Sephadex LH-20 column chromatography, using MeOH as eluent to give eleven fractions (Fr.1 –Fr. 11). Fractions (100 ml each) were collected, from which fractions 9 and 10 were pooled, evaporated to dryness, and further purified the active compounds by a preparative HPLC (C₁₈ reversed phase column, Prep Nova Pak, Waters) using H₂O–MeCN (60:40, v/v) as eluent, yielding 1 (40 mg) and 2(57 mg). Finally chemical structures of compound 1 and 2 Figure 1. were elucidated using UV, IR, MS and NMR spectroscopy. The spectroscopic data of racemosol (1) and demethylracemosol (2) were in agreement with those published in the literature (Anjaneyulu et al.,1986).



1. R= Me ; Racemosol
2. R= H; Demethylracemosol

Figure1. Structures of compounds racemosol and demethylracemosol

Human colon cancer cells (COLO 205 cell line)

COLO 205 (colon epithelial cell derived from ascites metastatic site) were incubated with Roswell Park Memorial Institute (RPMI) 1640 medium containing L-glutamine and phenol red, supplemented with 10% (v/v) fetal bovine serum, 0.1% penicillin-streptomycin and hepes (complete RPMI). The cell cultures were kept at a temperature of 37°C in humidified air containing 5% CO₂.

Mouse Fibroblast (L929) cell line

L929 (fibroblast derived from mouse adipose) were incubated with Dulbecco's Modified Eagle medium (DMEM), medium containing L-glutamine and phenol red, supplemented with 10% (v/v) fetal bovine serum, 0.1% penicillin-streptomycin and hepes (complete DMEM). The cell cultures were kept at a temperature of 37°C in humidified air containing 5% CO₂.

General experimental procedure

Melting points (m.p., °C) were measured with Electrothermal 9100. IR and UV spectra were acquired using Perkin-Elmer FT-IR 2000, FTIR system NICOLET 6700 and SHIMADZU UV-2600, respectively. H-NMR (500 MHz), C-NMR (125 MHz) and 2D-NMR spectra were performed using Bruker AV 500. NMR spectra were recorded on Bruker ARX-300 and ARX-600 spectrometers, used acetone-*d*₆ as solvents with TMS as the internal standard. ESI-MS and EI-MS 13 MS data were obtained by using Bruker microTOF spectrometer and Thermo Finnigan Polaris Q, respectively. Sephadex LH-20 (Pharmacia, Co.,

Switzerland), Prep. semi-preparative HPLC was carried out on C₁₈ reversed phase column, (250 mm × 10 mm, 10 µm, Prep Nova Pak, Waters) and SHIMADZU SPD-10A UV detector. Other chemicals and solvents in this experiment were all analytical grade or HPLC grade from J.T.Baker® Chemical Co.

Preparation of COLO 205 cells

COLO 205 cells were cultured on 75-cm² flasks and the culture medium was changed every 48 hours. For further experiments COLO 205 were sub-cultured at 85-100% confluence by using Trypsin-EDTA mixture to detach cells from the coated plate surface and passaged into 75-cm² flasks or onto 24 well plates for further *in vitro* study.

Investigation of the Cytotoxic activity of racemosol and demethylracemosol COLO 205 cells

Cell viability was measured by MTT assay. COLO 205 cells were treated with compounds 1-2 (5, 10, 20, 40, 80 and 160 µM) for 24 hours. L929 cells were dosed separately at concentrations of 0.5–10.0 µM with compounds 1-2.

Data and Statistical Analysis

The data were presented as mean ± SEM. The data was analyzed by one-way ANOVA followed by Dunnet's test. Prism Graph Pad 3 was used for statistical analysis. P<0.05 was considered significant.

RESULTS AND DISCUSSION

Racemosol (1) and demethylracemosol (2) were obtained from the root extract of *B. malabarica* after purification by Sephadex LH-20 column chromatography, followed by preparative HPLC (reversed phase C₁₈ column). The yields of compounds 1-2 were 0.0002%, 0.0003% (dry weight), respectively. The exact mass at m/z 340.1706, calcd. for C₂₁H₂₄O₄ obtained from the ESITOF mass spectrum of racemosol A (1) and at m/z 326.4013 calcd. for C₂₀H₂₄O₄ obtained from the ESITOF mass spectrum of demethylracemosol (2). The ¹H and ¹³C NMR spectra (Table 1) of racemosol (1) were generally similar to that of demethylracemosol (2).

Table 1.The ^1H and ^{13}C NMR spectra of compounds 1-2

C	Racemosol		Demethylracemosol	
	δC , mult. ^a	δH , mult., J in Hz	δC , mult.	δH , mult., J in Hz
2	73.27, s		73.54, s	
3	22.18, t	3.03, dd, (12.0, 6.0)	22.60, t	3.10, dd, (6.9, 3.6)
		3.38, dd, (12.0, 6.0)		3.38, dd, (9.4, 6.0)
4	31.70, d	4.50, m, (12.0, 6.0)	32.55, d	4.52, m, (4.5, 2.7)
4a	137.56, s		137.16, s	
5	130.20, s		129.43, s	
6	115.00, d	6.71, dd, (9.1, 12.0)	115.76, d	6.78, d, (6.0)
7	111.90, d	6.70, dd, (12.0, 6.0)	108.60, d	6.71, d, (7.5)
8-OCH ₃	143.50, s		146.43, s	
8-OH				7.25, s
9-OH	141.50, s	7.35, s	143.07, s	7.80, s
10	113.50, s		114.43, s	
11	37.50, t	3.10, m, (16, 6.3)	38.68, t	3.10, m, (13, 16.6)
		2.10, m, (16, 6.3)		2.40, t, (13, 16.6)
12	33.80, t	2.74, dt, (13, 16.6)	34.65, t	2.74, dt, (13, 5, 16)
12		3.20, dt, (13, 16.6)		3.20, dt, 16.6
13	135.83, s		137.16, s	
14	118.90, d	6.10, s	115.76, d	6.10, s
15-OH	151.90, s	5.60, s	153.13, s	5.60, s
16	108.50, s		108.60, s	
16a	153.50, s		154.31, s	
16-CH ₃	8.60, q	2.02, s	8.52, q	1.95, s
OCH ₃	60.00, s	3.80, s		
Gem-Me	23.30, q, 30.31, q	1.34, s, 1.53, s	23.22, q, 30.38, q	1.31, s, 1.49, s

Racemosol, (1,7,8,12b-Tetrahydro-2,2,4-trimethyl-10-methoxy-2H-benzo [6,7] cyclohepta [1,2,3-de][1]benzopyran-5,9-diol); deep red prisms, m.p. 200° C, IR ν_{max} (ATR) cm^{-1} : 3450, 2980, 1560, 1220, 1085.

Demethylracemosol, (1,7,8,12b-Tetrahydro-2,2,4-trimethyl-2H-benzo[6,7] cyclohepta[1,2,3-de][1]benzopyran-5,9,10-triol); deep red crystals, m.p. 217-218° C, IR ν_{max} (ATR) cm^{-1} : 3450, 2980, 1580, 1500, 1270, 1080.

Effects of racemosal and demethylracemosal mouse fibroblast (L929) cell line

From bioassay-guided fractionation of the root extract of *B. malabarica* after, racemosal and demethylracemosal significantly suppressed COLO 205 production in a dose-dependent manner without cytotoxic effect (0 – 60 μM) excepts for the concentration of 80 μM that showed cytotoxicity (Fig. 2).

Effects of racemosal on cellular viability of COLO 205

COLO 205 cells were incubated with racemosol at concentrations 0, 2.5, 5, 10, 20 and 40 μM for 24 hours. The result showed that there was a notable decrease in mean percentage of cell viability from 100% (0 μM) to 6% (10, 20 and 40 μM racemosol) (Fig. 3). Additionally, racemosol at greater than 10 μM showed the percentage inhibition about 95%

with no significantly different from each other. This experiment demonstrated that racemosol was toxic to COLO 205 cells at increasing concentrations and the IC_{50} of racemosol was 6.16 μ M (Fig.4).

Effects of demethylracemosol on cellular viability of COLO 205

COLO 205 cells were exposed to demethylracemosol at concentrations of 0, 2.5, 5, 10, 20 and 40 μ M for 24 hours. The result from this experiment demonstrated that there was a gradual reduction in mean percentage of cell viability from 100% (0 μ M demethylracemosol) to approximately 95% (10, 20 and 40 μ M demethylracemosol) (Fig. 5). In addition, this experiment demonstrated that demethylracemosol was toxic to COLO 205 cells at increasing concentrations and the IC_{50} of demethylracemosol was 6.45 μ M (Fig.6).

Effects of racemosol and demethylracemosol on normal mouse fibroblasts (L929)

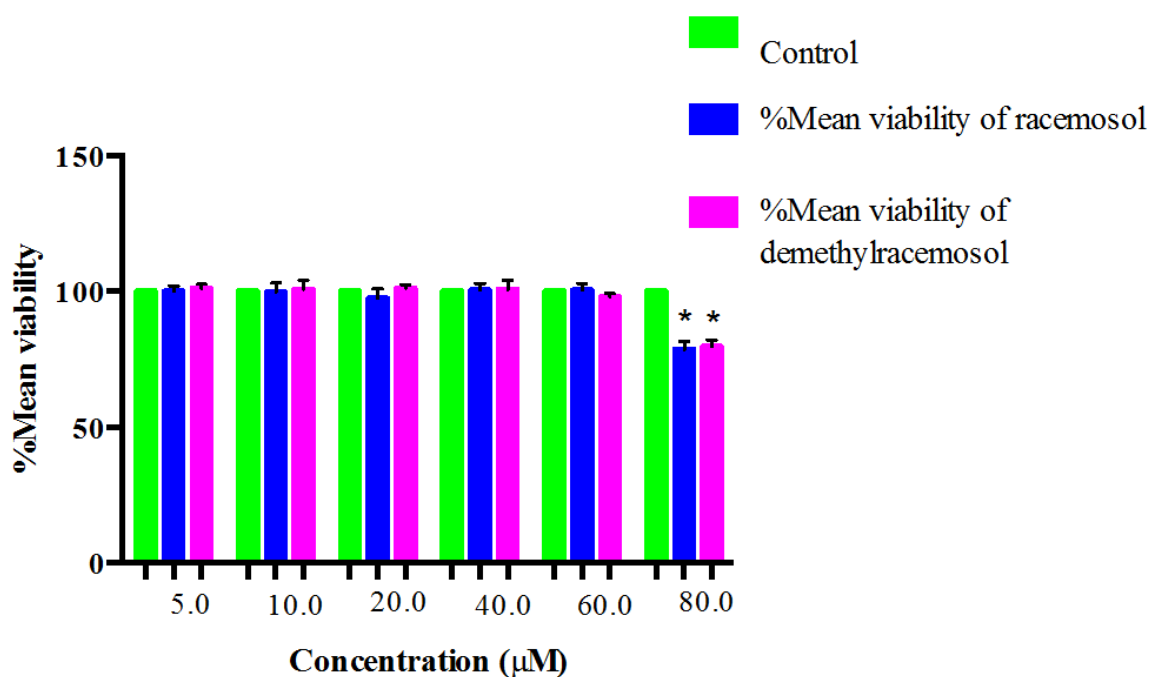


Figure 2. Bar chart illustrated mean percentage of cell viability and standard deviation of L929 cells against racemosol and demethylracemosol at increasing concentrations after 24 hours incubation. N = 3, * $P \leq 0.05$ vs. untreated cells.

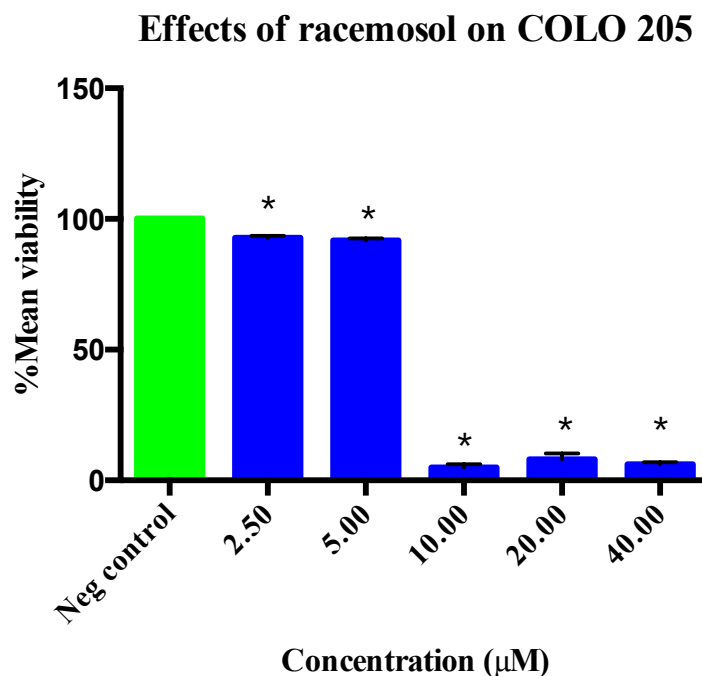


Figure 3. Bar chart illustrated mean percentage of cell viability and standard deviation of COLO 205 cells against racemosol at increasing concentrations after 24 hours incubation. N = 6, ****P≤0.0001 vs. untreated cells (0 µM racemosol).

Log-dose inhibition curve of racemosol on COLO 205

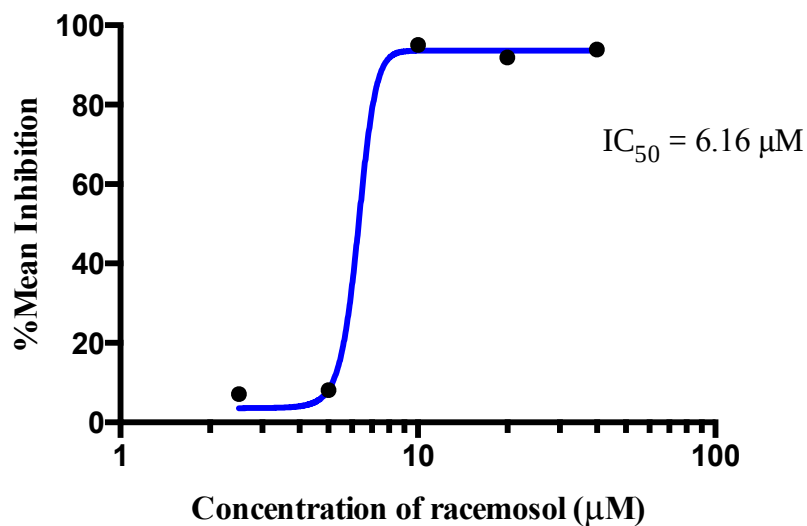


Figure 4. Dose-inhibition curve showed 50% inhibition concentration of racemosol on COLO 205 cells at 6.16 µM.

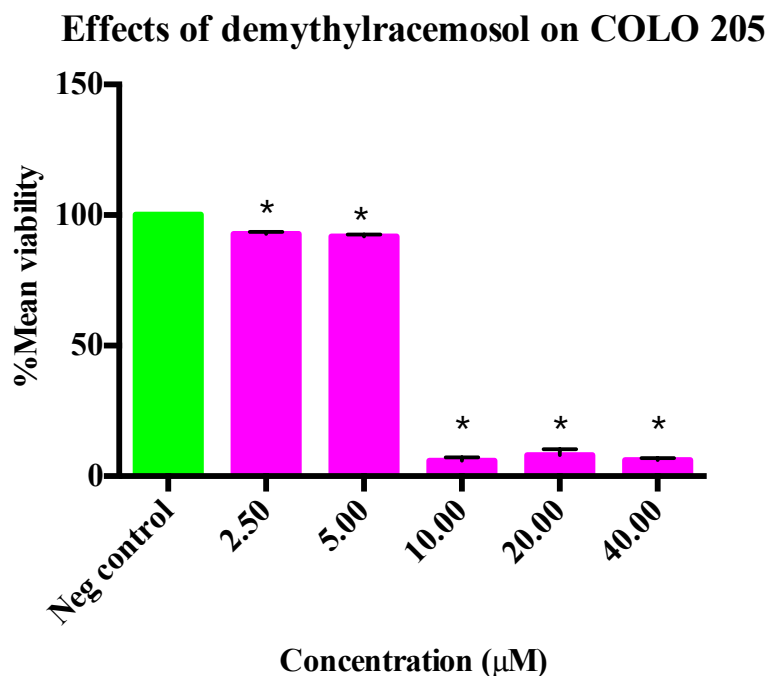


Figure 5. Bar chart illustrated mean percentage of cell viability and standard deviation of COLO 205 cells against demethylracemosol at increasing concentrations after 24 hours incubation. N=6, ****P≤0.0001 vs. untreated cells (0 μM demethylracemosol).

Log-dose inhibition curve of demethylracemosol on COLO 205

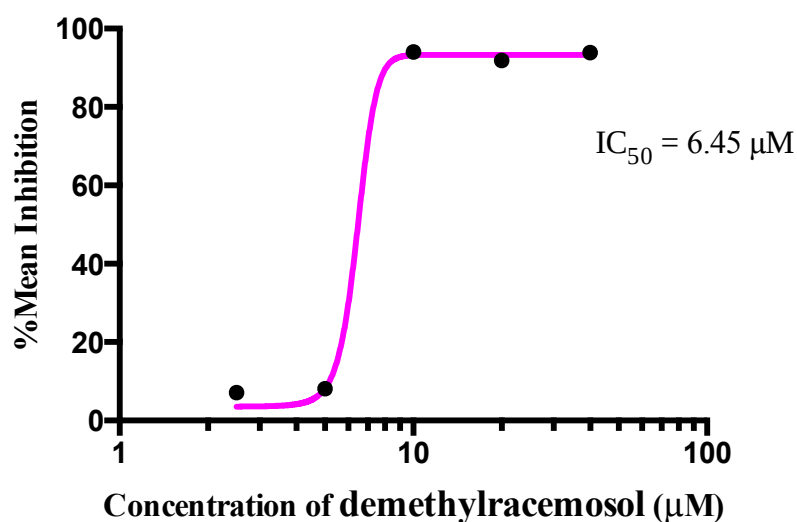


Figure 6. Dose-inhibition curve showed 50% inhibition concentration of demethylracemosol on COLO 205 cells at 6.45 μM.

Log-dose inhibition curve of racemosol and demethylracemosol on COLO 205

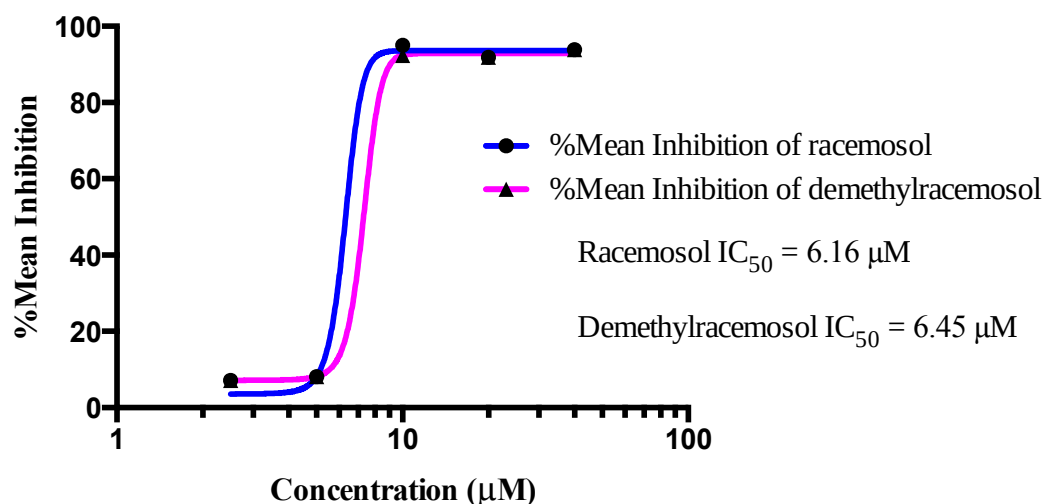


Figure 7. Dose-inhibition curve showed 50% inhibition concentration of racemosol compared to demethylracemosol against COLO 205 cells.

CONCLUSION

This investigation proved that constituents extracted from the roots of *B. racemosa*, racemosol and demethylracemosol showed a significant cytotoxic activity on COLO 205 cells with the IC₅₀ of 6.16 and 6.45 μM respectively.

ACKNOWLEDGMENTS

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