

**Cell growth inhibitory action of an unusual labdane diterpene,
13-*epi*-sclareol in breast and uterine cancers *in vitro***

Koneni V. Sashidhara^{a*}, Jammikuntla N. Rosaiah^a, Abdhesh Kumar^a, Hemant K Bid^b,
Rituraj Konwar^b, Naibedya Chattopadhyay^b,

^a*Division of Medicinal and Process Chemistry, ^b Division of Endocrinology,*
Central Drug Research Institute, Lucknow, India

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*Corresponding author's address: E-mail: sashidhar123@rediffmail.com .Fax: +91-0522-
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ABSTRACT

In the course of our studies on the isolation of bioactive compounds from the roots of *Coleus forskohlii*, a traditional herb in India, rare 13-*epi*-sclareol has been isolated, and its structure determined by extensive 2D NMR. And also this is the first report of isolation from this plant. Isolated compound showed anti-proliferative activity in breast and uterine cancers *in vitro*. The anti-proliferative activity of 13-*epi*-sclareol is comparable with Tamoxifen in terms of IC₅₀ and also showed concentration dependent increased apoptotic changes in breast cancer cell line, MCF-7.

INTRODUCTION

Breast cancer is the most prevalent form of cancer in women world over (Parkin *et al.*, 2005). Estrogen induced mammary cell proliferation is implicated as the first step in the development of breast cancer. Estrogen receptor (ER) positive proliferative phase is generally followed by estrogen independent tumor growth, which subsequently metastasizes. Tamoxifen, a selective estrogen receptor modulator (SERM) is the drug of choice for inhibiting estrogen action in ER+ breast cancer, whereas chemotherapeutics are suggested for advanced stages (Lawrence and Hartmann, 2003). Uterine cancer is also an important endocrine cancer in women. Natural products have traditionally remained a good source of agents with anti-cancer activity. However, proper characterization particularly from the viewpoint of cancer specific action devoid of possible cytotoxic effects of naturally derived compounds on normal cells is essential. In case of breast cancer, isolating compounds with selective anti-cancer activity and comparing its efficacy with known molecules like Tamoxifen is a standard approach for drug development.

Coleus forskohlii Briq., a perennial dried herb of Labiatae growing in India as well as Yunnan Province of China, and it has been used since ancient times for medical treatment in Ayurvedic traditional medicine (Gabetta *et al.*, 1989). Forskolin, a major labdane diterpene isolated from the roots of this herb is capable of preventing tumor colonization and metastasis (Agarwal and Parks, 1983) and inhibiting growth and inducing apoptosis of myeloid and lymphoid cells (Gutzkow *et al.*, 2002; Moon and Lerner, 2003; Taetle and Li-en, 1984). Recently, it has been found that forskolin, a potent PP2A activator (Feschenko *et al.*, 2002; Neviani *et al.*, 2005), induced marked apoptosis, reduced proliferation, impaired colony formation, inhibited tumorigenesis, and restored differentiation of BCR/ABL-transformed cells regardless of their degree of sensitivity to imatinib (Neviani *et al.*, 2005).

Sclareol a diterpene isolated from fruits of *Cistus creticus* is shown to inhibit growth and cell cycle progression of human leukemic cell and induces apoptosis. Sclareol exerts antiproliferative activity by reducing c-Myc without altering bcl-2 expression (Dimas *et al.*, 1999). Sclareol is also reported to be non-cytotoxic to resting human peripheral blood mononuclear leukocytes (Dimas *et. al.*, 1999) and have LD₅₀ > 5 g/kg in rats (Food and Chemical Toxicology, 1992).

In the course of our studies on the isolation of bioactive compounds, we purified 13-*epi*-sclareol, a labdane diterpene from roots of *Coleus forskohlii* (Fig.1). Recently, this compound was shown to have antibacterial activity against gram-positive bacteria (Tapia *et al.*, 2004). As 13-*epi*-sclareol was isolated with a yield of 0.2% and it has never been subjected to systematic pharmacological evaluation, we considered it as an appropriate candidate for investigation for its anti-cancer properties. In the present study we carried out experiments to investigate the antiproliferative and pro-apoptotic activity of the isolated compound in MCF-7 and Ishikawa cell line. In addition, we have evaluated its cytotoxicity in primary osteoblast cell and Vero cell line. Our study reveals specific anti-cancer activity of 13-*epi*-sclareol in breast and uterine cancer *in vitro*.

MATERIALS AND METHODS

Isolation of compounds

The roots of *Coleus forskohlii* were collected by the Botany department, and voucher specimen has been maintained. The roots were extracted with 8 L of ethyl alcohol four times in a percolator. The resultant alcoholic extract was combined and concentrated under reduced pressure to give 200g of alcohol extract. This was fractionated with hexane, chloroform and n-butanol successively. The resultant chloroform fraction (20g) was subjected for conventional silica gel column chromatography and eluted with mixtures of

increasing polarity of hexane: ethyl acetate solvent system to give 13 *epi*-sclareol and were characterized by using $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, 2D NMR, IR and Mass Spectral data and comparing with literature data (Stierle *et al.*, 1988).

Cell line

MCF-7 is a breast cancer cell line originally derived from pleural effusion (Soule *et al.*, 1973), was used for screening of anti-cancer breast activity. Ishikawa is uterine endometrial adenocarcinoma cell line that expresses estrogen and progesterone receptor (Nishida, 2002), was used to evaluate the activity of the compound in uterine cancer. Vero cell line, derived from normal kidney of African green monkey (Yasumura and Kawakita, 1963), and primary osteoblast cells were used for evaluation of cytotoxicity in non-transformed cells.

Cell Viability Assay

Loss of cell viability was assessed by the trypan blue exclusion method. Cells treated with or without were harvested by trypsinization. After incubation in 0.04% trypan blue (Sigma-Aldrich) for 4 min, cells were counted under a hemocytometer. The number of cells that retained the dye (nonviable) and the total cell number were noted.

Antiproliferative activity Assay

The drug antiproliferative activity of 13-*epi*-sclareol was determined using MTT assay (Mosmann, 1983). 1×10^4 cells/well were seeded in 100 μl DMEM, supplemented with 10% FBS in each well of 96-well microculture plates and incubated for 24 h at 37 $^{\circ}\text{C}$ in a CO₂ incubator. Compounds, diluted to the desired concentrations in culture medium, were added to the wells with respective vehicle control. After 48 h of incubation, media were removed and to each well 100 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) was added and the plates were further incubated for 4 h.

Supernatant from each well was carefully removed, formazon crystals were dissolved in 100µl of DMSO and absorbance at 540 nm wavelength were recorded.

Apoptosis

Cells were seeded at a density of 10,000/well in 12-well cell culture plate over already placed 60-mm dishes containing One day later, the medium was replaced, and cells were treated with 10 and 20 µM of 13-*epi*-sclareol. Cells treated with vehicle (0.001% DMSO) were included as controls. After overnight treatment, Hoechst 33342 (Sigma-Aldrich) were added to medium at 0.5ug/ml, respectively. After incubation for 30 min at 37°C, cells was wet mounted and image were captured with fluorescence microscope (Leica DMLB, Wetzlar, Germany).

RESULTS AND DISSCUSSION:

13-*epi*-sclareol exhibited comparable anti-proliferative activity in breast cancer cell line MCF-7 cells with IC₅₀ of 11.056 µM as compared to 14.34 µM of Tamoxifen (Fig. 2). Tamoxifen is a SERM and it modulates breast cancer cell growth mostly via ER beside other mode of action. Sclareol has already been reported to have antiproliferative property with IC₅₀ lower than 20µg/ml and down regulate c-Myc in leukaemic T-cell (Dimas et. al., 1999). Our study reveals that the epimer of sclareol also possesses antiproliferative activity in breast cancer cells. 13-*epi*-sclareol also showed anti-proliferative activity in Ishikawa cell line where Tamoxifen failed to show any such effect (Fig. 3). This finding is in line with earlier reported activity of Tamoxifen in uterine cancer cell line (Hochner-Celnikier *et al.*, 1997). This indicates that the mode of anti-proliferative activity of 13-*epi*-sclareol may be different from Tamoxifen. In addition, the cellular viability in response to 13-*epi*-sclareol in time-dependent manner characterized with trypan blue exclusion dye showed similar inhibition profiles in both MCF-7 and Ishikawa cell line (Fig. 4 and 5).

Next, cell viability in response to 13-*epi*-sclareol was assessed in Vero cell line and in primary osteoblast cells by MTT assay. 13-*epi*-sclareol exhibited lesser cytotoxicity in vero cell (Fig. 6) and primary osteoblast cells in comparison to taxol (Fig.7). Our data indicate that 13-*epi*-sclareol may have a favorable toxicological outcome over other cytotoxic anti-cancer agents.

The 13-*epi*-sclareol treated MCF-7 cells were next subjected to Hoechst-3347 nuclear stain to study apoptotic changes. Its epimer sclareol is already known to cause apoptosis (Dimas *et al.*, 1999). Our data show that 13-*epi*-sclareol in the concentrations of 10 μ M and 20 μ M showed concentration dependent increased apoptotic changes (Fig. 8).

In conclusion, 13-*epi*-sclareol demonstrates promising anti-proliferative activity against endocrine cancer cell lines viz., MCF-7 and Ishikawa and induces concentration dependent cell death. Its activity is comparable to Tamoxifen and devoid of cytotoxicity in normal cells viz., Vero cell line and primary osteoblast cells. Further studies are necessary to assess its mode of action and *in vivo* efficacy.

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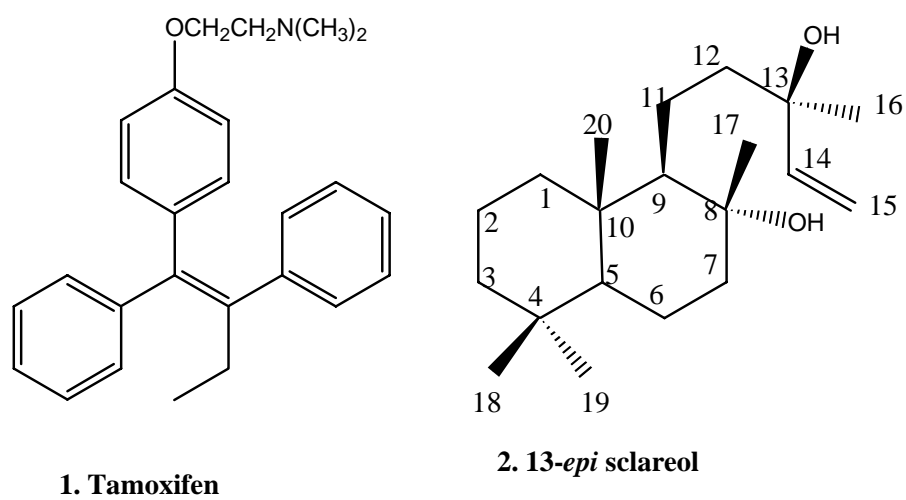


Figure 1 1. Synthetic known antibreast cancer agent
2. Naturally occurring compound.

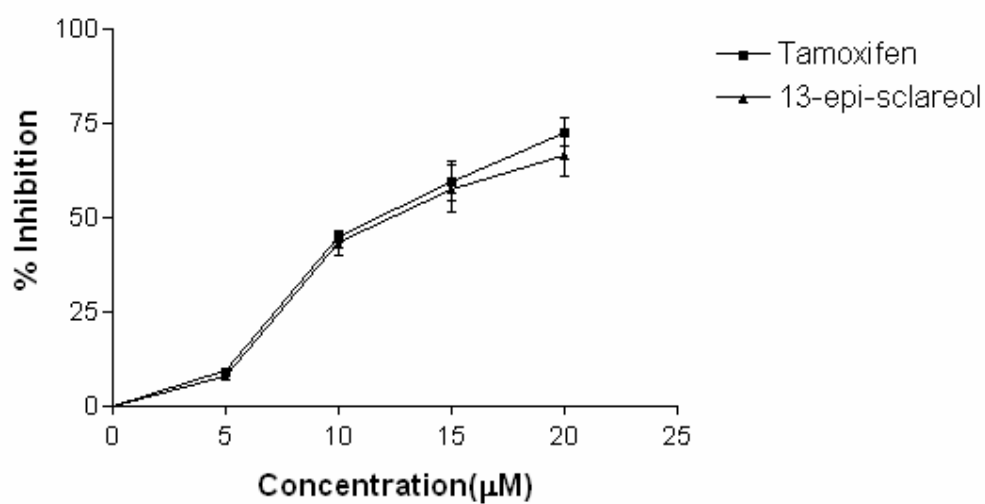


Figure 2: Dose-dependant inhibition of MCF-7 cells by 13-epi sclareol. MCF-7 cell were incubated overnight with 5, 10, 15 and 20 µM 13-epi sclareol and percentage of cell growth inhibition was determined with MTT assay. Tamoxifen was used as positive control (n=9, $P>0.05$, two-tailed student's *t*-test).

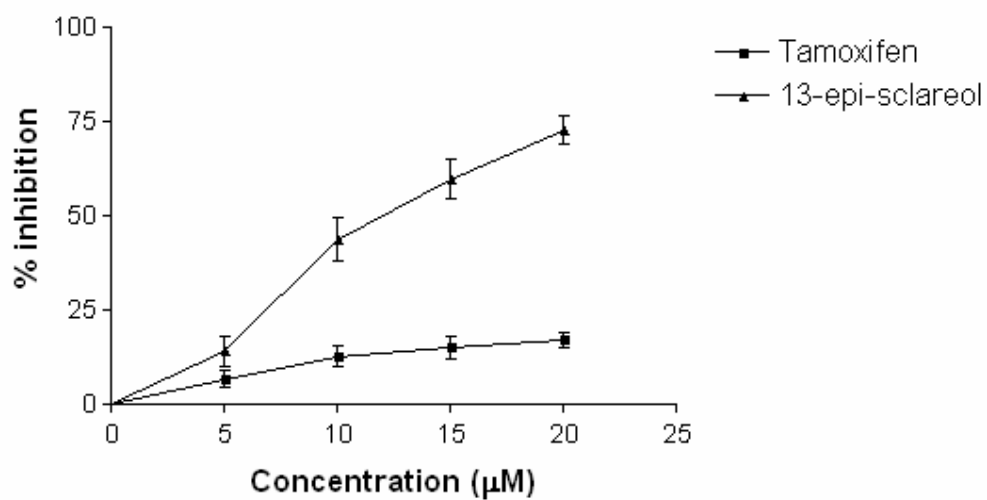


Figure 3: Dose-dependant inhibition of endometrial adenocarcinoma (Ishikawa) cells by 13-epi sclareol. Ishikawa cells were incubated overnight with 5, 10, 15 and 20 µM 13-epi sclareol and percentage of cell growth inhibition was determined with MTT assay. Tamoxifen was used as positive control (n=9, $P<0.05$, two-tailed student's *t*-test).

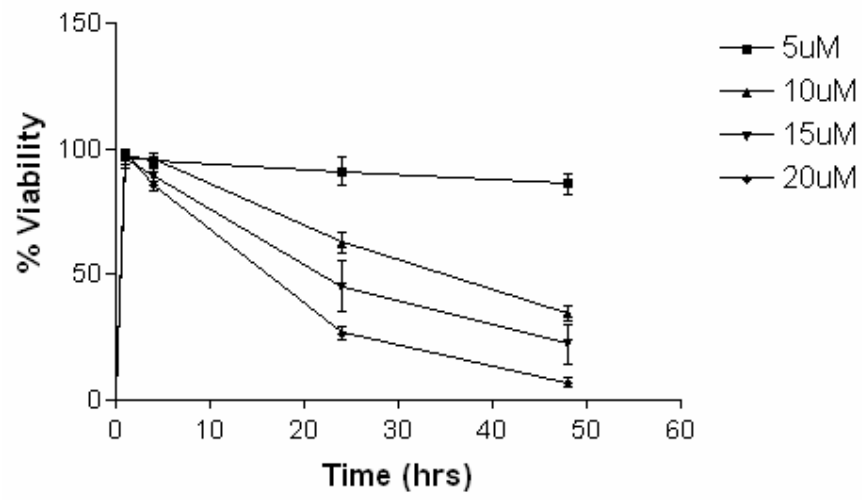


Figure4: Time-dependant inhibition of MCF-7 cells by 13-epi sclareol. MCF-7 cells were incubated for 1, 4, 24 and 48hrs with different concentrations of 13-epi sclareol and percentage viability was determined with trypan blue cell viability assay (n=9, $P<0.05$, two-tailed student's t -test).

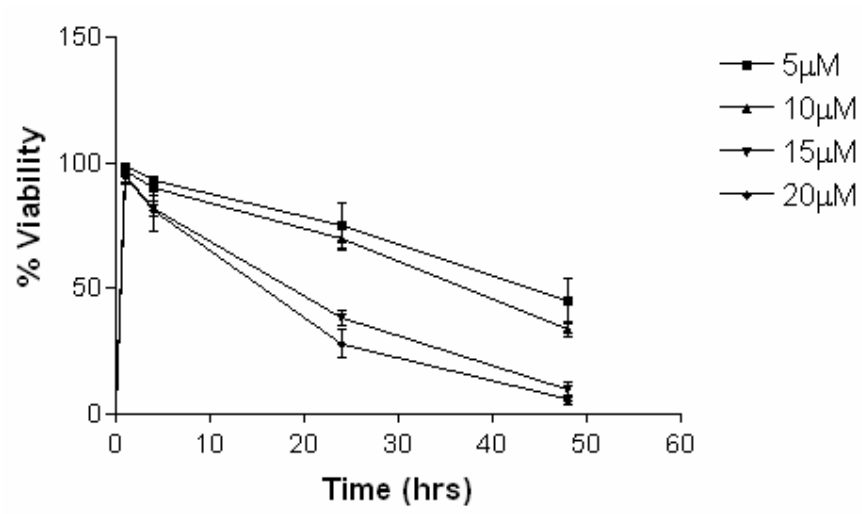


Figure 5: Time-dependant inhibition of endometrial adenocarcinoma (Ishikawa) cells by 13-epi sclareol. Ishikawa cells were incubated for 1, 4, 24 and 48hrs with different concentrations of 13-epi sclareol and percentage viability was determined with trypan blue cell viability assay (n=9, $P < 0.05$, two-tailed student's *t*-test).

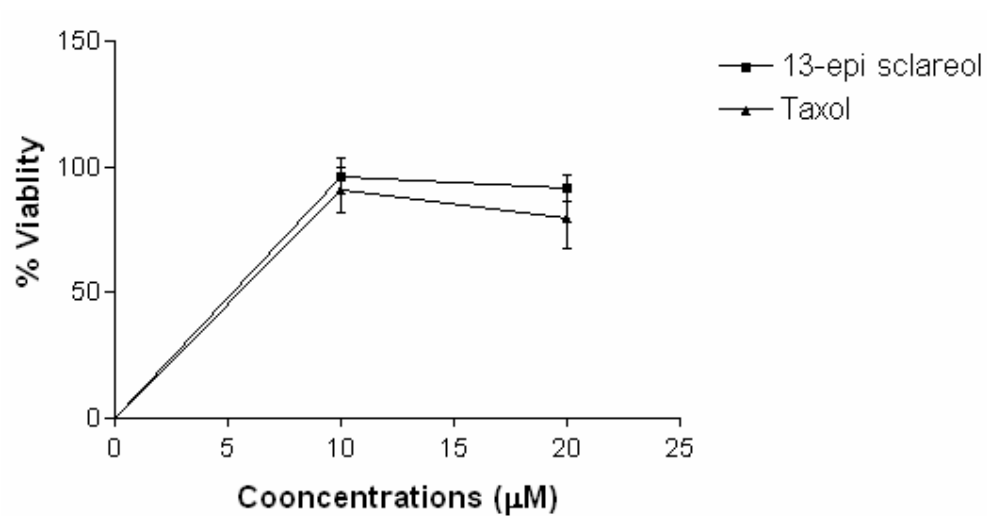


Figure 6: 13-epi sclareol does not show cytotoxic effect in normal cell line (vero). Cells were incubated 12 hours with 10 and 20 µM of 13-epi sclareol and percentage viability was determined with MTT assay (n=9, $P < 0.05$, two-tailed student's *t*-test).

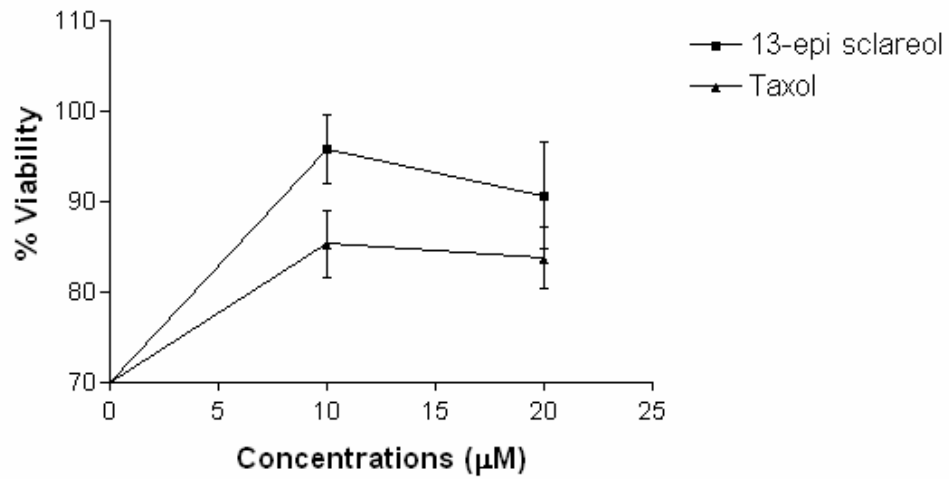


Figure 7: 13-epi sclareol does not have cytotoxic effect in primary osteoblasts. The osteoblasts were culture from d1 rat calvaria and incubated for 12 hours with 10 and 20 µM of 13-epi sclareol and percentage viability was determined with MTT assay (n=9, $P<0.05$, two-tailed student's *t*-test).

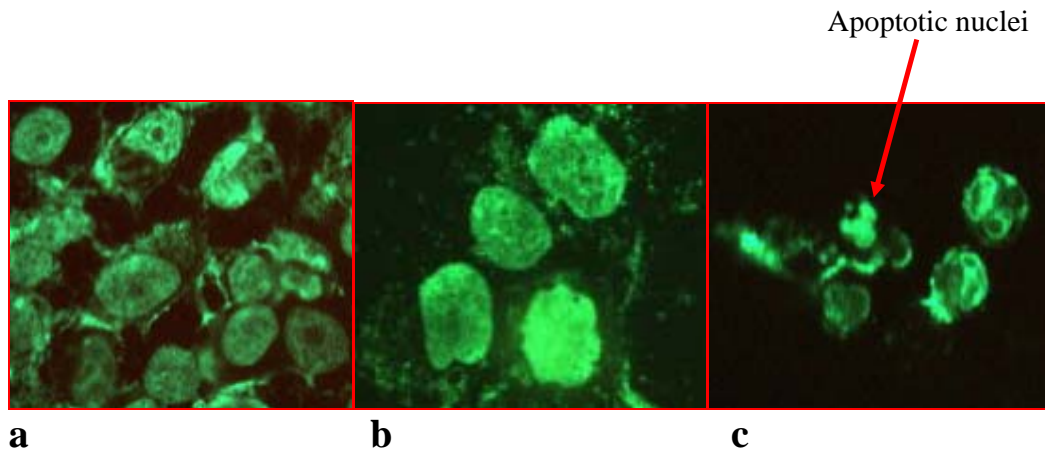


Figure 8: 13-epi sclareol induces apoptosis in MCF-7 cells. Cells were treated with different concentration of 13-epi sclareol and stained with Hoechst-3347 as described in materials and method (a- untreated control, b-10 μ M 13-epi sclareol and c-20 μ M 13-epi sclareol).