Sohag Journal of Science
An International Journal

http://dx.doi.org/10.18576/sjs/030101

Antimicrobial and Antiproliferative activity of Tomenin compound isolated from Seriphidium Herba-Alba

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Received: 19 Oct. 2017, Revised: 22 Jan. 2018, Accepted: 18 Feb. 2018.
Published online: 1 Jan. 2018.

Abstract: *Seriphidium herba-alba* (Asso) Sojak *(Asteraceae* family) (previously named *Artemisia herba-alba*) is a dwarf shrub fast growing in arid and warm climates and muddy areas, used in folk medicine for the treatment of gastric disturbances such as diarrhoea, abdominal cramps and for healing external wounds. Tomenin was isolated from *Seriphidium herba-alba*. The structure of isolated compound was identified by the extensive spectroscopic techniques such as 1D-NMR and EI-MS. Isolated compound was subjected to antiproliferative potential against cancer cell lines of the liver (HepG-2), colon (HCT-116) and breast (MCF-7). Viability was assessed by the standard colorimetric assay using the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). The assay is based on the reduction of the yellow, water-soluble MTT to purple water-insoluble formazan crystals in the mitochondria of living cells. MTT assay can give a first idea of the impact of a treatment on cell viability and provides therefore general information on cytotoxicity. Additionally, the total extract of *S. herba alba* and isolated purified compound were tested for antimicrobial activity. Tomenin exhibited low inhibitory effect against *F. solani* only and all other tested micro-organisms was able to resist it. *Seriphidium herba-alba* total extract has good inhibitory activity against all tested pathogenic bacteria.

Keywords*:* *Seriphidium herba-alba*, Tomenin, HepG-2, HCT-116, MCF-7, MTT.

1Introduction

The genus *Artemisia* L. (Asteraceae) contains upwards of 500 species, is the largest genus in the tribe Anthemideae, and one of the largest genera in the family[1,2]. Species in this genus often dominate dry regions of the mid to high latitudes of the Northern Hemisphere. *Artemisia* species are widely distributed in the temperate and subtropical areas of the Northern hemisphere, and only a few species can be found on the southern hemisphere [2]. The most important center of diversification of *Artemisia* is central Asia, Europe, northern America and South Africa. The Mediterranean region and Northwest America are two secondary speciation areas. Much of the western United States is dominated by sagebrush ecosystems where *Artemisia* species serve as a source of food for various types of animal wildlife [3,4]. *Artemisia* is a highly evolved genus with a wide range of life forms, from tall shrubs to dwarf herbaceous alpine plants, occurring in a variety of habitats between Arctic alpine or montane environments to the dry deserts [2,5]. Some of the better known species can be recognized by common names such as sagebrush, mugwort, wormwood, absinthe, armoise, sweet Annie, etc. *Artemisia* species are predominantly wind-pollinated and can produce copious amounts of pollen. In areas where they grow in abundance, *Artemisia* spp. can therefore be a major cause of allergies in humans [6, 7]. After various taxonomic rearrangements, the genus was divided into five large groups; *Absinthium* DC., *Artemisia* L., *Dracunculus* Besser, *Seriphidium* Besser and *Tridantatae* [1]. This classification is however not accepted by all authors. Some of these genera (such as *Seriphidium*) are considered authors as members of *Artemisia,* but others are usually regarded as Independent [8]. *Seriphidium* was separated from *Artemisia* as new genus [9,10]. *Artemisia* species produce terpenes, diterpenes, triterpenes, polyacetylenes, sesquiterpene lactones, flavonoids, coumarins and glycosides [11,12] and many of these chemical compounds have been found to have biological activity. The most widely known and successfully marked compound is artemisinin, obtained from *Artemisia annua*. In combination with other compounds artemisinin is currently one of the most

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effective treatments against malaria, till 2009, eighty countries have officially adopted artemisinin-based combination therapies as their first line of treatment against malaria Artemisinin has also been shown to induce apoptosis in cancer cells, selectively kill cancer cells *in vitro*, and retard the growth of implanted fibro sarcoma tumors in rats [13]. The aim of the current research is the phytochemical study of the isolated compounds from *S. herba-alba*, by extraction, separation and describes the content of active substances. As well as, structure elucidation of the isolated compound by means of spectroscopic data analysis. The research work also focused on the evaluation of *S. herba-alba* total extract and Tomenin for the activity *in vitro* against the pathogenic strains, evaluate the cancer chemopreventive activity of Tomenin.

2 Experimental

2.1 General Experimental Procedures

^{1}H- and ^{13}C- NMR spectra were recorded in CD_3OD on a JEOL ECA-600 spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C, respectively). The chemical shift (δ) are given in ppm with a TMS as an internal standard and a coupling constants (J) reported in Hz. EI-MS was performed on a Finnegan LCQ ion trap mass spectrometer. EI-MS experiments were performed using a (Thermo ISQ Single Quadrupole system). High performance liquid chromatography (HPLC) was performed on an Agilent pump equipped with an Agilent-G1314 variable wavelength UV detector at 254 nm and a semi-preparative reverse-phase column (Econosphere™, RP-C_{18}, 5 µm, 250 × 4.6 mm, Alltech, Deerfield, IL, USA). Silica gel 60 (230–400 mesh) was used for column chromatography. Pre-coated silica gel plates (Kieselgel 60 F_{254}, 0.25 mm) were used for TLC analyses. Spots were visualized by heating after spraying with 10% H_2SO_4.

2.2 Plant Material

Seriphidium herba-alba was collected in June 2014 from South Sinia (Saint Catherine), Egypt and a voucher specimen SH-1101 has been deposited in the herbarium of St. Katherine protectorate, Egypt.

2.3 Extraction and Isolation

The dried powder of an aerial parts of *S. herba-alba* (2.5 kg) was extracted with CH_2Cl_2–MeOH (1:1) at room temperature. The extract was concentrated in *vacuo* at 45°C to obtain a residue of 200 g of a dark brown residue. The residue was fractionated on a silica gel column (6×120 cm) eluting with *n*-hexane (3 L) followed by a gradient of *n*-hexane:CH_2Cl_2 up to 100% CH_2Cl_2 and CH_2Cl_2–MeOH up to 50% MeOH (3 L of each solvent mixture). The CH_2Cl_2:MeOH (1:1) fraction (12.5 g) was subjected to a second silica gel column (3×120 cm) eluted with CH_2Cl_2:MeOH:H_2O (5:3:1) generating two subfractions. Subfraction 1A (3.3 g) was further purified by HPLC eluted with MeOH:H_2O (15: 85). The flow rate was set to 1.5 mL/min and was at 0–70 min to afford Tomenin (33 mg, purity > 90% by HPLC), (eluent CH_2Cl_2/MeOH/H_2O 5:3:1, R_f = 0.25).

2.4 Bioassay

2.4.1 Antimicrobial Assay

The antibacterial and antifungal properties of the purified compound **1** in addition to the SHA-total extract were tested *in-vitro* against pathogenic bacteria, yeast and fungi in comparison with control drug Thiophenicol (Thiamphenicol, Sanofiaventis, France) as an antibacterial agent, and Treflucan (Fluconazole, Egyptian International Pharmaceutical Industries Company. EIPICO) as an antifungal agent, by using the agar diffusion technique in Petri dishes according to the reported method [14].

The isolated compound and the total extract of *S. herba-alba* were tested against Gram- positive bacteria (*Bacillus subtilis* ATCC6633 and *Staphylococcus aureus* ATCC29213), Gram-negative bacteria (*Escherichia coli* ATCC25922), yeast (*Candida albicans* ATCC10321), and fungi (*Fusariumsolani* NRC15). Bacteria and yeast strains are American Type culture collection and fungal isolates were obtained from the culture collection of the Department of Chemistry of Natural and Microbial Products, National Research Center, Cairo, Egypt.

Preparation of paper discs:

The isolated compound Tomenin and the total extract were mounted on a paper disc prepared from blotting paper (5 mm diameter) on a concentration of (100µg/5µl DMSO /disc). Total extract was mounted on a concentration of (1mg/5µl DMSO/disc). Thiophenicol and Treflucan were used as a positive control for antibacterial and antifungal activity in a concentration of (100µg/disc). DMSO used as a negative control.

Preparation of agar plates:
Spores suspension of pathogenic strains were prepared and adjusted to be approximately (1×10^6 spores^{-} ml of fungi and 1×10^8 of bacteria). 1 ml of fungal and bacterial spore suspensions was inoculated into each plate containing 50 ml of sterile PDA and nutrient agar medium respectively.
Application of the discs:
After the media had cooled and solidified, the discs were applied on the surface of the inoculated agar plates and left for 30 min at 4 ºC for compounds diffusion. The plates were incubated for 24 h at 30 ºC for bacteria and 72 h at 28 ºC for fungi. Diameters of zones of inhibition produced around the discs were measured in (mm) at three different points and the average values are reported as Mean ± SD using MS Excel.
Minimal inhibitory concentration (MIC)
The purified Tomenin was evaluated for its minimal inhibitory concentration MIC at the final concentrations; 100, 50, 25 and 12.5 µg. And the total extract was tested at different concentrations of 1000, 500, 250, 125 and 62.5µg. The lowest concentration showing inhibition zone around the disc was taken as the minimum inhibitory concentration (MIC).

2.4.2 Antiproliferative of Isolated Compounds

Chemicals Used:

Dimethyl sulfoxide (DMSO), MTT and trypan blue dye was purchased from Sigma (St. Louis, Mo., USA). Fetal Bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza.

Cell lines:

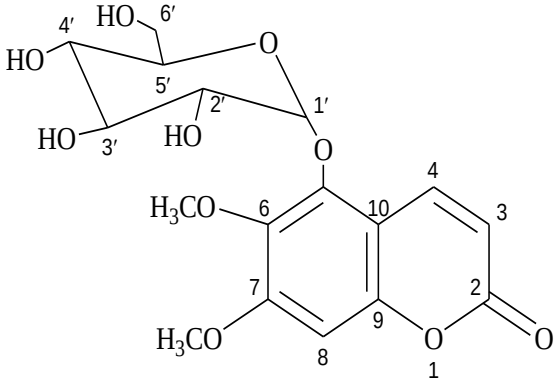
MCF-7 cells (human breast cancer cell line), **HepG-2** cells (human Hepatocellular carcinoma) and **HCT-116** (colon carcinoma) were obtained from the American Type culture collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50µg/ml gentamycin. The cells were maintained at 37 ºC in a humidified atmosphere with 5% CO_2 and were sub cultured two to three times a week.
Cell viability assay:
For antitumor assay, the tumor cell lines were suspended in a medium at concentration $5x10^4$ cell/well in Corning® 96-well tissue culture plates, and then incubated for 24 h. The tested compounds were then added into 96-well plates (three replicates) to achieve twelve concentrations for the tested compound and total extract. Six vehicle controls with media or 0.5 % DMSO were run for each 96 well plate as a control. After incubating for 24 h, the numbers of viable cells were determined by the MTT test. Briefly, the media was removed from the 96 well plate and replaced with 100 µl of fresh culture RPMI 1640 medium without phenol red then 10 µL of the 12 mM MTT stock solution (5 mg of MTT in 1 mL of PBS) to each well including the untreated controls. The 96 well plates were then incubated at 37°C and 5% CO_2 for 4 hours. An 85 µl aliquot of the media was removed from the wells, and 50 µl of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37 °C for 10 min. Then, the optical density was measured at 590 nm with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [1-(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA) [15,16].
Statistical Analysis
All results are expressed as mean ± standard deviation (SD). Statistical differences between correlated samples were evaluated using Graph pad Prism software (San Diego, CA. USA).

3 Results and Discussion

3.1 Structure Elucidation

Tomenin was isolated as a colourless oil from the (CH_2Cl_2: MeOH) (1:1) fraction of *Seriphidium herba-alba* extract. EI-MS gave a molecular ion peak $[M]^+$ at *m/z* 384.87 (0.75%) which solved for $C_{17}H_{20}O_{10}$ and $[M\text{-}C_6H_{10}O_5]^+$ ion peak at *m/z* 191.70 (24.24%), this indicated the sequential loss of hexose moiety, base peak at *m/z* 42.94 (100%) which represent C_2H_2O, this indicated the presence of a methoxy group in its mass spectrum. The ^{1}H-NMR spectral data of isolated compound showed the presence of common features characteristic for tri-substituted coumarin derivatives. A coumarin skeleton was evident in isolated compound based on the AB spin system for the two doublet proton at δ_H 6.33 and 7.88

ppm, which is characteristic of the H-3 and H-4 protons of coumarins [17]. The ^{1}H-NMR spectrum revealed the presence of a typical AB system for H-3 and H-4, the presence of a pair of doublets at (δ_H 6.33 and 7.88, J = 9.6 Hz) attributed to protons at positions 3 and 4. The presence of one-proton singlet in the aromatic region at (δ_H 6.99, 1H, s, H-8) pointed to the occurrence of a tri-substituted benzenoid ring. Two of these substituents were identified as a methoxy groups at positions 6 and 7 at (δ_H 3.87, 3 H, s, C-6- OCH_3) and (δ_H 4.00, 3H, s, C-7-OCH_3). The remaining substituent was identified as a glucopyranosyloxy unit with β-configuration based on the anomeric signal at (δ_H 5.18, 1H, d, J=8.28 Hz, H-1'; δ_C 102.86, C-1'). ^{13}C-NMR spectrum revealed the presence of unequivalent 17 carbons (Table **1)**. Nine signals appeared in the region (δ_C 102.86 - 162.28) ppm represent nine carbon atoms of coumarin nucleus including, three methine groups and six quaternary carbons, six signals in the region at (δ_C 102.86, 74.33, 77.20, 70.02, 76.80, 61.43) were assigned to Glu-C-1′ to C-6′, respectively. Two signals at (δ_C 55.76, 61.20) ppm confirmed the presence of two methoxy carbons in the molecule. Supporting evidence for the arrangement of the methoxy groups on the coumarin nucleus which was available from ^{13}C-NMR spectral data [18].The chemical shift of one methoxyl group at (δ_C 61.20), clearly established the presence of a substituent in the ortho position. On the other hand, the second methoxyl group should be flanked by at least one unsubstituted carbon at (δ_C 55.76). Signals for aromatic carbons appeared at (δ_C 144.52, C-9) and (δ_C 104.79, C-10). The downfield appearance of C-9 compared to C-10 is due to its neighboring position to an oxygen atom. The above information led to characterization of the isolated compound as Tomenin. The NMR and EI-MS data showed good agreement with previously published data [17-23].



Chemical structure of Tomenin

3.2 Invitro Antibacterial and Antifungal Assay

The agar diffusion assay of Tomenin and *Seriphidium herba-alba* total extract demonstrated different antimicrobial effects against tested micro-organisms as shown in **Table 2**. The results indicated that Tomenin exhibited low inhibitory effect against *F. solani* and all other tested micro-organisms were able to resist it. *Seriphidium herba-alba* total extract has good inhibitory activity against all tested pathogenic bacteria with zones of inhibition range from 9 to 11 mm and (MIC) in range from 125 to 500 µg. In addition the total extract showed inhibitory effect against the fungal pathogen *F. solani* and *C. albicans* with zone of inhibition 10 and 9 mm, respectively.

3.3 Antiproliferative Activity

To evaluate the antiproliferative activity of Tomenin, their anti-proliferative potential against MCF-7 cells (human breast cancer cell line), HCT-116 (colon carcinoma) and HepG-2 cells (human Hepatocellular carcinoma) were assessed using a cell viability assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were treated at concentrations 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.6 µg/mL, 7.8 µg/mL and 3.9 µg/mL for 24 h. The anticancer drug, Doxorubicin, was used as a positive control. Tomenin showed cytotoxic activity for all the cancer cells tested (MCF-7, HCT-116 and HepG-2) with Half maximal inhibitory concentration (IC_{50}) values, obtained from dose-response curves are (327 ± 6.7, 409 ± 8.6, 300 ± 5.9), respectively (**Figure 3**).

Table 1: ^{1}H (600MHz) and ^{13}C-NMR (150MHz) spectral data of Tomenin recorded in CD_3OD.

Position	δ_H (*J* in Hz)	δ_C
H-2	----------	161.20 (C)
H-3	6.33 d (9.6)	114.46 (CH)
H-4	7.88 d (9.6)	141.02 (CH)
H-5	----------	142.12 (C)
H-6	----------	115.37 (C)
H-7	----------	150.15 (C)
H-8	6.99 s	102.86 (CH)
H-9	----------	144.52 (C)
H-10	----------	104.79 (C)
6-OCH_3	3.87 s	61.20 (CH_3)
7-OCH_3	4.00 s	55.76 (CH_3)
GLu -H-1′	5.18 d (8.28)	102.86 (CH)
GLu -H-2′	3.64 m	74.33 (CH)
GLu -H-3′	3.42 m	77.20 (CH)
GLu -H-4′	3.36 m	70.02 (CH)
GLu -H-5′	3.28 m	76.80 (CH)
GLu -H-6′	3.47 m, 3.73 m	61.43 (CH_2)

Table 2: Anti-microbial activity (diameters of growth inhibition zones) of Tomenin and *S. herba-alba* extract against the pathogenic strains.

compounds	Inhibition zone diameter (mm)				
	Gram positive		Gram	Yeast	Fungi
	B. subtilis	*S. aureus*	*E. coli*	*C. albicans*	*F. solani*
Tomenin	N.A.	N.A.	N.A.	N.A.	7±0.00
S. herba-alba total	10±0.71	11±0.71	9±0.70	9±0.70	10±1.40
Thiophenicol	20±0.70	18±1.42	15±0.70	N.A.	N.A.
Treflucan	N.A.	N.A.	N.A.	22±0.42	12±0.71

Notes: The agar diffusion technique was followed and the inhibition zone diameter (IZD) expressed in (mm).

Thiophenicol and Treflucan were used as positive controls at a concentration of 100 µg/disk. N.A. No activity.

Table 3: Minimum inhibitory concentration of Tomenin and *S. herba-alba* extract against the pathogenic strains.

	Minimum Inhibitory Concentration MIC (µg/disc)				
	Gram positive		Gram negative	Yeast	Fungi
compounds	*B .subtilis*	*S. aureus*	*E. coli*	*C. albicans*	*F. solani*
Tomenin	-	-	-	-	100
S. herba-alba - *total extract*	125	250	500	500	62.5
Thiophenicol	3.13	3.13	25	-	-
Treflucan	-	-	-	12.5	50

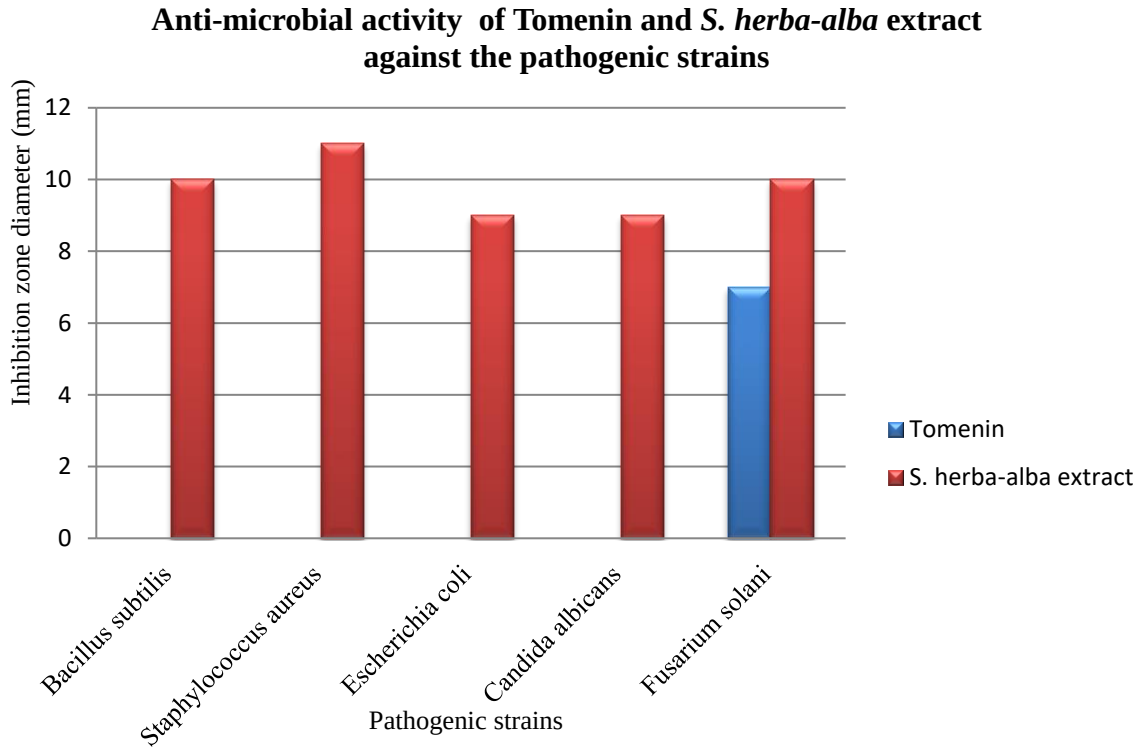


Fig. 1: Anti-microbial activity of Tomenin and *S. herba-alba* extrac against the pathogenic strains.

4 Conclusions

Seriphidium herba-alba afforded Tomenin which is the first report of the isolation of Tomenin from *S. herba-alba.* It has not antibacterial properties and has a weak antifungal and antiproliferative properties.

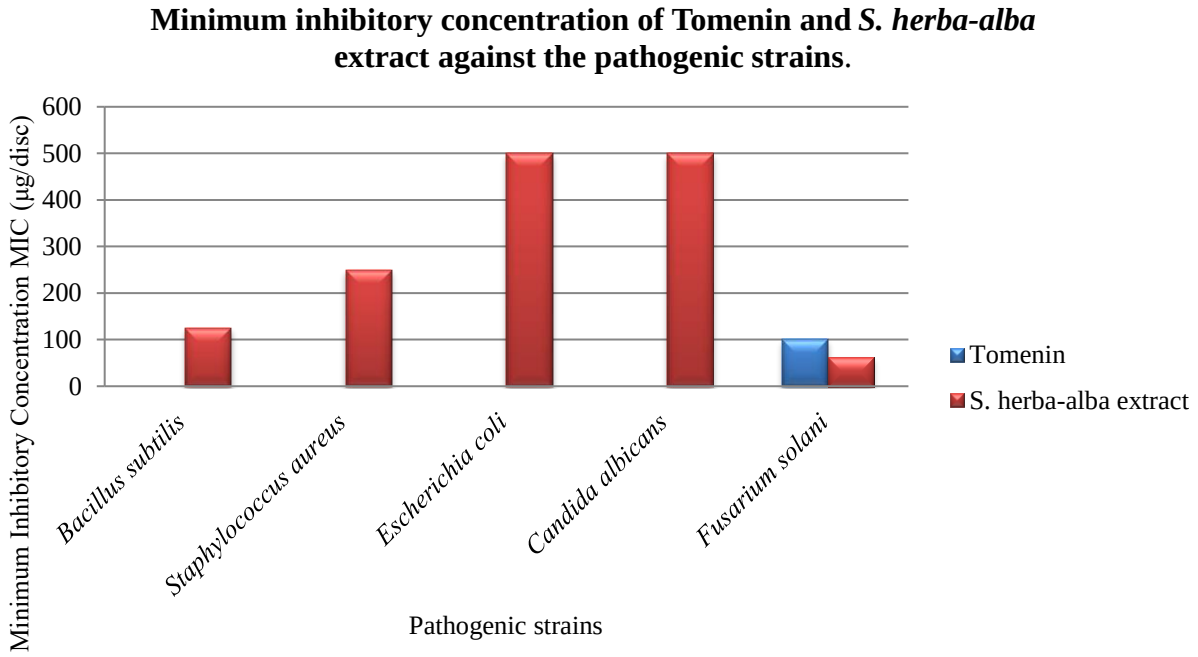


Fig. 2: Minimum inhibitory concentration of Tomenin and *S. herba-alba* extract against the pathogenic strains.

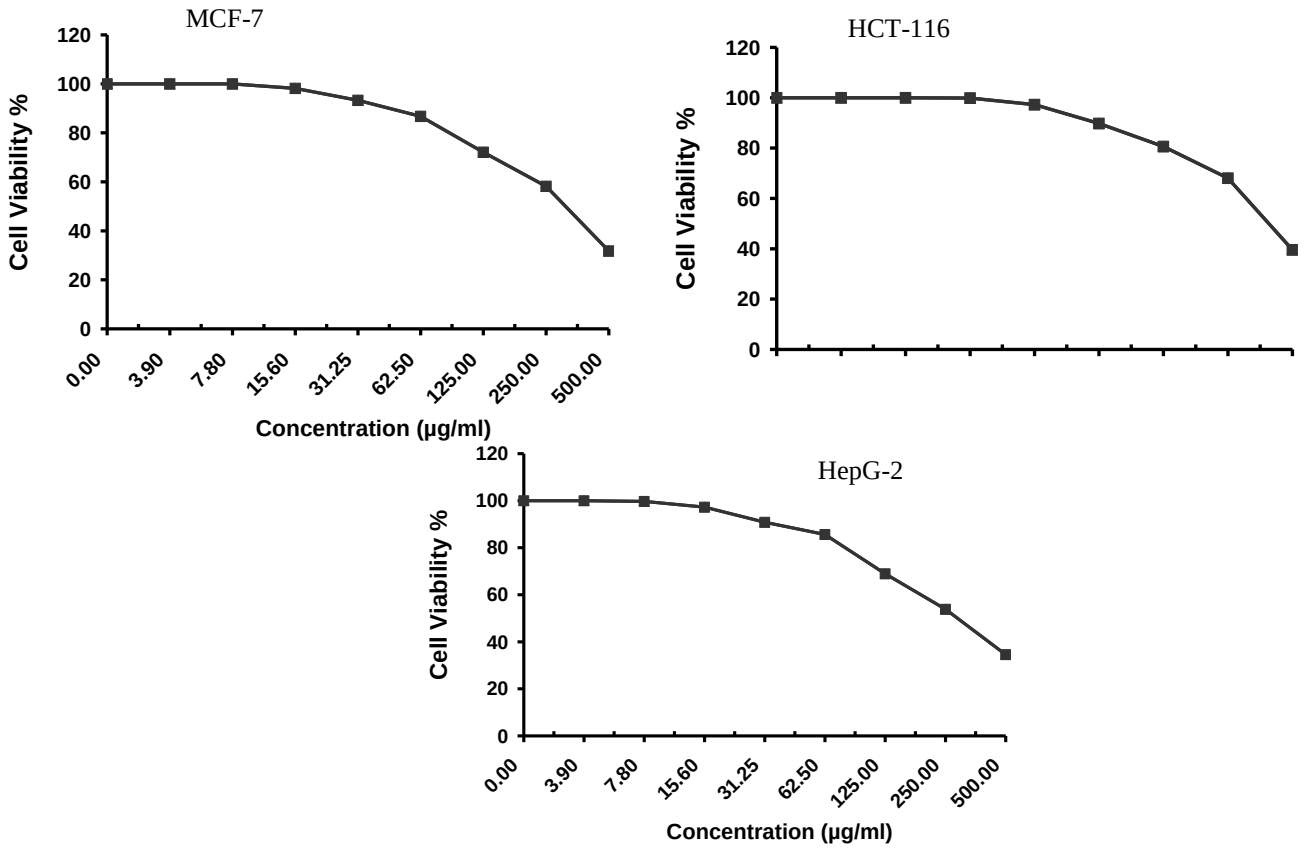


Fig.3: Relationship between different doses of Tomenin (3.9–500 µg/mL) and the cell viability of **MCF-7**, **HCT-116** and **HepG-2** using MTT assay.

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