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Extraction and chemoprofiling study of *Eulophia herbaceae* Lind. tubers for colorectal cancer

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Abstract

Colon cancer is a great evildoer behind mortality around the world. Some non-developed regions like China and India stand in the third position in this regard. Maximum of the presently accessible colon cancer therapeutic choices are exorbitant and come up with hazards. However, traditionally available herbal derivatives or medications are comparatively innocuous. One prominent example is β-sitosterol (BS), derived from *Eulophia herbaceae* Lind. (EH), exhibiting antineoplastic potential against malignancies associated with colon, lung, breast, *etc*. The ultimate goal of the present research was to isolate and explore the impact of BS from EH tubers. BS has been extracted and isolated from tubers' metabolic extract (MTE), further characterized by UV, IR, TLC, 1HNMR, GC-MS and 13C NMR techniques. Further, validation of pharmacological activity by employing sulforhodamine B (SRB) cytotoxicity assay on (COLO-205) confirmed its anticancer potential. The BS demonstrated > 80 g/ml of lethal concentration value (LC_{50}) as well as a total growth inhibition value (TGI). The Concentration of average growth inhibition (GI_{50}) for BS was less than 21.0 µg/ml against a cell line that has been found to possess significant anticancer potential against human colon cancer (COLO-205). From the present research, it can be concluded that the tubers of EH containing BS possess paramount anticancer potential against human colon cancer and can be considered for further development as biomedicine.

1. Introduction

According to WHO (2021), 1 million people died in 2020 due to colon cancer (CC). The modern therapeutic strategies for managing human colorectal carcinoma include surgeries, chemo and radiation treatment. However, the prevalent approaches are still unsatisfactory. Hence, there is a need for a more optimized compound (Xie *et al.*, 2020). Many compounds isolated from plant sources revealed anticancer activity, induced the proliferation of cancer cells, and led to apoptosis (Awad *et al*., 2000). BS is utilized as an essential biomarker derived from EH for managing CC in humans (Abdel-Hamid *et.al*., 2017). The Orchidaceae family is amongst the most prominent families of flowering plants and several members of the genus *Eulophia* belonging to the family Orchidaceae are utilized as excellent health promoters (Chandrashekhar *et al*., 2018). Traditionally, EH tubers were utilized for a variety of applications, including tumour treatment of the scrofulous gland, appetite stimulation, aphrodisiac and therapeutic interventions for cardiac ailments (Tatiya *et al*., 2013). According to the evidence, numerous hibiscus species exhibit significant anticancer activity on cancerous cells (Alam *et al*., 2018). Due to the steroidal lipophilic nature of BS, it is available in several vegetable and plant oils (Ameen *et al*., 2015). Structurally, it is similar to cholesterol but with an additional ethyl group at 24th carbon, as illustrated in Figure 1. It has an absolute oral bioavailability of 0.41%, an 85 ml per hour clearance, 46l distribution volume, along with a 5.8 mg/day turnover, which is significantly smaller than cholesterol's (Bin Sayeed *et al*., 2016)

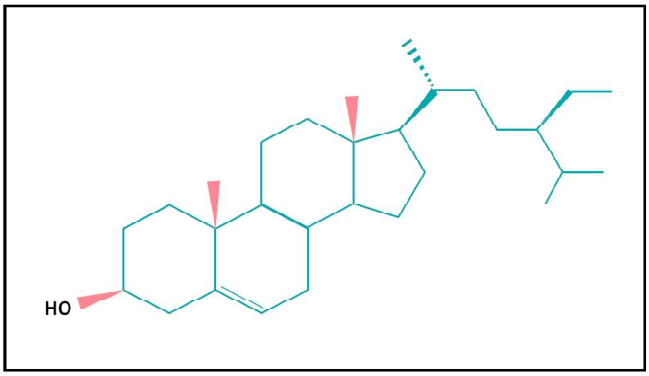

Figure 1: Basic structure of β-sitosterol.

Several investigational studies demonstrated that BS forbids the proliferation of several tumor cell lines related to the initiation of the cell cycle arrest and sphingomyelin cycle (Awad *et al*., 2000; Devi *et al*., 2018). Some of the researchers stated that the various signalling pathways, the cell cycle, mortality, growth, survivability, invasion, angiogenesis, metastasis and inflammation are also severely affected by BS. However, the fact that it is believed to be innocuous often contradicts all of the cancer chemotherapeutics currently accessible (Ameen *et al*., 2015). Ovesna *et al*. (2016) documented the experimental suppression of colon and metastatic breast carcinoma proliferation by taraxasterol and BS. They claimed that these components could influence multiple stages of tumorigenesis, such as their antagonistic effect on the formation, stimulation and induction of malignant cells, as well as prevention

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of tumour cell invasion and propagation (Lkovaâ *et al*., 2004). A significant decrease in the expression of b-catenin and proliferating antigen cell nuclear (markers of proliferation) was observed due to BS administration in CC. In the study of CC, BS acts as an efficacious agent to lessen *in vivo* oxidative stress and serves as a chemo-protective medium in CC (Baskar *et al.*, 2010). The investigations were predicated upon the lowering levels of catalase, glutathione S-transferase, glutathione peroxidase, superoxide dismutase, glutathione reductase and glutathione by 1, 2-dimethylhydrazine (DMH) in the hepatic and colon region of an animal model. BS was able to effectively protect the organism of the animals from this damage (Awad *et al*., 2000).

Different chromatographic techniques have been utilized to isolate and purify BS from a variety of plant species and sources, such as the tubers of EH.The standard process of isolation involves the preparation of a MTE from a selected plant, then executing various chromatographic separations over the silica gel column and managing the fractions by TLC (Chandrashekhar *et al*., 2018).With the modernization of instrumental advanced analytical techniques, it became possible for scientists to recognize BS along with other phytosterols in the presence of cholesterol to analyze the qualitative as well as quantitative aspects of several body mechanisms, including humans, animals, cells and tissues. Although, BS is extensively studied globally, the research on BS from tubers of EH for CC is in infancy. There is a lack of studies on isolation, optimization, analytical method and colorectal cancer potential. Hence, in the present study, efforts were undertaken to alienate and structural elucidation of BS using a suitable method, as well as to assess its antitumor activity utilizing a sulforhodamine B(SRB) cytotoxicity assay against CC (Colo-205).

2. Materials and Methods

2.1 Materials

The EH tubers were collected from the Toranmal hills Nandurbar, Maharashtra, India.The successful identification and authentication of collected EH tubers were done by Dr. S.R.Kshirsagar, Botanist from Shri Shivaji Vidya Prasarak Sanstha's Late.Karamveer Dr. P.R.Ghogrey Science College, Dhule, Maharashtra, India. Herbarium specimen prepared and submitted to institute number RCPIPER/PCOG/MH2016-29. All the chemicals and reagents used to conduct the present research were procured from Loba Chem India and were of analytical grade purity.

2.2 Methods

2.2.1 Methanolic extract preparation

Initially EH, tubers were washed with the water to remove dirt and debris and cut into small pieces, washed with 90% ethanol to inhibit microbial growth and degradation of plant material throughout the drying and storage process. Dried tubers were pulverized and passed through mesh 40. Dried tubers were powered by a pulveriser and passed through sieve no 40**.** The processed plant material was stored in an airtight container with appropriate labelling and used for further analysis. 500 g of powdered tubers of EH was packed in a thimble containing filter paper and initially extracted with methanol having high polarity in several batches using the Soxhlet apparatus. The extracts were separated, concentrated under reduced pressure in a rotary vacuum evaporator, filtered using whatmann filter paper and subjected to drying. Later, the dried extracts were collected and kept in a desiccator for further analysis. The extractive value of MTE was calculated. Phytoconstituent was isolated by fractionating the methanol extract into the pet. ether. Pet. ether fraction (PEF) contained 4 spots that preparative TLC further isolated; the exact procedure is discussed below (Harborne, 1984; Mukherjee, 2002).

2.2.2 Preparative TLC isolation

The MTE was analyzed phytochemically and proceeded for isolation of active ingredients. Where PEF contained 4 spots on the TLC plates. Further fractionation was required, which was achieved by preparative TLC plates. Bands of the samples were applied and run in TLC chambers. Post-development of the plate, the band was separated and, in the forms, strip it was scrapped out and collected separately. For recollecting samples, de-sorbing in a suitable solvent was performed, and TLC was used for purity check. Further, samples were subjected to spectroscopic analysis as discussed in the below sections (Wagnerand Bladt,1996).

2.3 Phytochemical screening

The MTE of tubers of EH was investigated for phytochemicals as per the standard procedures discussed below; the Liberman burchard and Salkowoski indicated the steroid presence.

2.3.1 Preliminary tests

For U. V spectral analysis compound's absorption spectrum was recorded in the range of 200-800 nm in ethanol on a 1 cm path length using UV spectroscopy (Shimadzu). The compound's IR spectrum was also recorded on FT-IR 8400-S using the liquid sampling technique. The TLC of the isolated compound was performed with merk HPTLC plate using Pet. ether: Acetone (8:2) as mobile phase with anisaldehyde H_2SO_4 as visualizing agent.^{1}H NMR was recorded at 400 MHz, and^{13}C NMR was recorded on spect 5 mm-PABBO BB (400 MHz) in $CDCL_3$ (Khandelwal, 2006).

2.3.2 HPTLC analysis (Wagnerand Bladt, 1996)

For HPTLC analysis, 10 mg of MTE was dissolved in a 10 ml analytical grade methanol, *i.e.*

1 µg/µl. Compared to spot application, specimen administration in bands offers higher resolution; Linomat V allows sample implementation (more than 2-12 µl) over narrow areas of HPTLC plate using a spraying strategy. The previously coated aluminium plates (10 x 10 cm) were employed as follows:

i. The plate was developed within Pet. ether: Acetone (8:2) as the solvent system in a C among twin troughs TLC chamber with an 8 cm covering.

ii. After development, plates were air-dried and subjected to derivatization.

iii. Derivatization of the plate was done by dipping the plate into anisaldehyde H_2SO_4 and finally air-dried.

iv. Scanner 3 was used for chromatogram evaluation.

v. The plate was scanned at 254 nm. Retention factor R_f and area under the curve (AUC) for each sample were determined using win CATS software (Version 1.3.0).

2.3.3 Anticancer activity using SRB assay(Kirtikar and Vichai 2006; Fang *et al.*, 2007)

SRB assay was executed on the CC cell line (COLO-205) to investigate the BS's anticancer potential from the MTE of EH tubers. A brief view of the method utilized is presented in Figure 2 below. Cell lines were cultured in the initial stage using RPMI 1640 media with 10% foetal calf serum and 2 m L-glutamine. Based on the doubling time associated with cellular lines, the cells were inoculated in 96 well microtiter plates for the screening experiment at plating densities of 100 µl. Post cell inoculation, at 37%, 5 % CO_2, 95% air and 100 % RH for 24 h, the microtiter plates were incubated before the addition of investigational drugs.

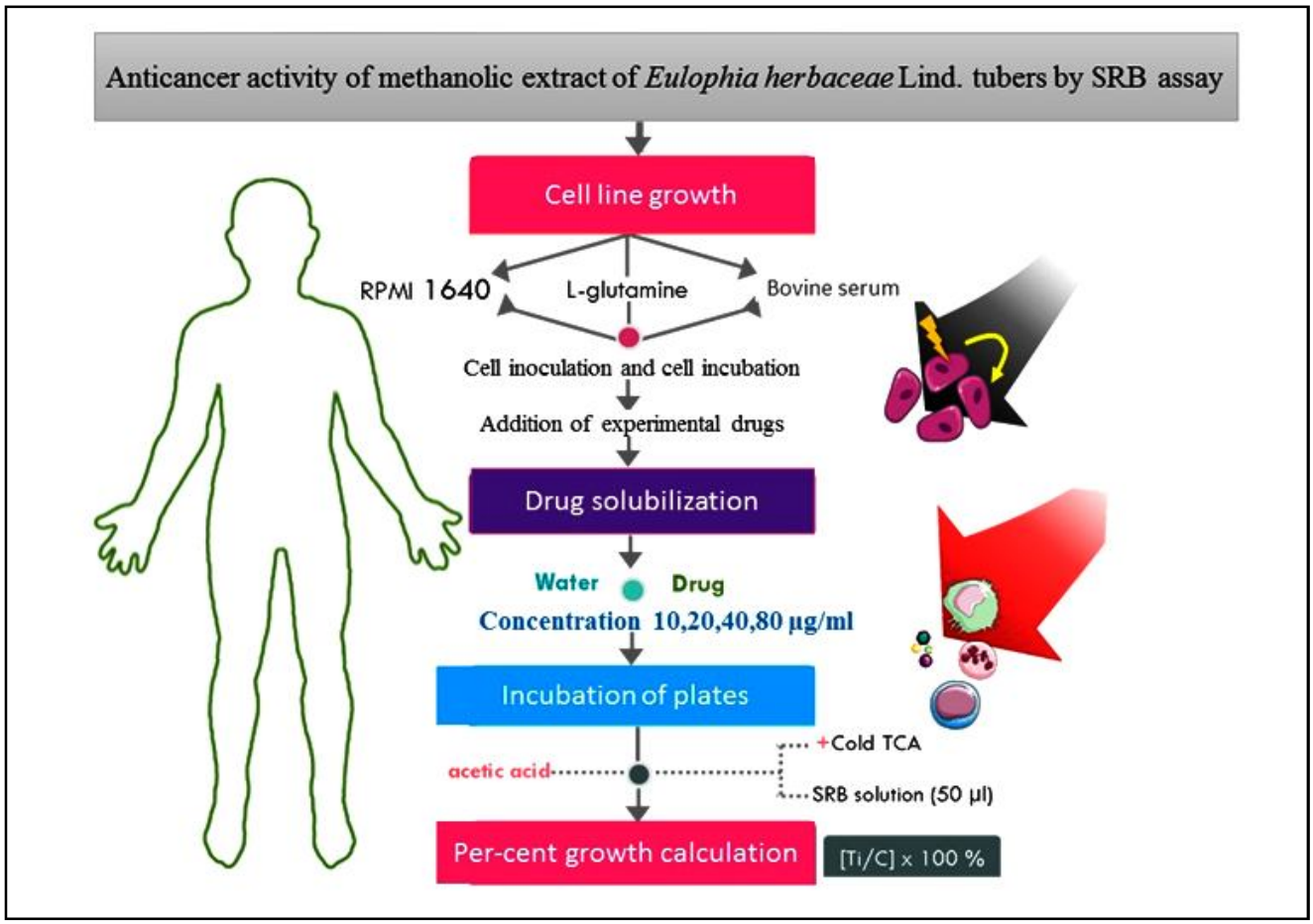

Figure 2: Anticancer activity using SRB assay.

A secondary phase was initiated wherein an appropriate solvent at 100 mg/ml, the investigational drug was solubilized and neutralized with water to 1 mg/ml and kept in a refrigerator before use. The 1 mg/ml frozen concentrate aliquots were thawed and diluted to 100-800 µg/ml with the entire media of the test component just at the instance of drug addition. The final drug concentrations, *i.e.*, 10- 80 µg/ml, were accomplished by combining 10 µl aliquots of each dilution with 90 µl media in microtiter wells. Plates were incubated immediately after adding the chemical under specific conditions for 48 h, and the assay was terminated by adding 50 µl chilled (frozen) TCA (30% w/v). The cells were fixed *in situ* and incubated at 4°C for 60 min. The supernatant was disposed of and washed 5 times with demineralized water, allowing air drying. The 50 µl of SRB solution [0.4 % (w/v)] was added in 1 % acetic acid (ATA), kept in each well and incubated for 20 min at room temperature. Post staining, the unbound stain was extracted and the remaining was eliminated by rinsing 5 times with ATA and subjected to air drying. The conjugated stain was repeatedly eluted with a 10 mm trizma base and recorded the absorbance using a plate reader at 540 nm with a standard λ_{max} 690 nm. The per cent inhibition was calculated on a plate-by-plate basis for experimental wells compared to control wells. It was assessed for every drug concentration using the six measurements and calculated using the following formula:

%Inhibition = [(Cell control – Experimental) 100×]/(cell control)

3. Results

3.1 Methanolic extraction and isolation

The methanolic extract of EH further fractionated with petroleum ether fraction; the compound was isolated, the petroleum ether fraction showed the presence of four brown to black spots, further separated by preparative TLC. This technique identified the single compound with an Rf value of 0.52 and labelled it compound-1(COMP-1), as shown in Figure 3.

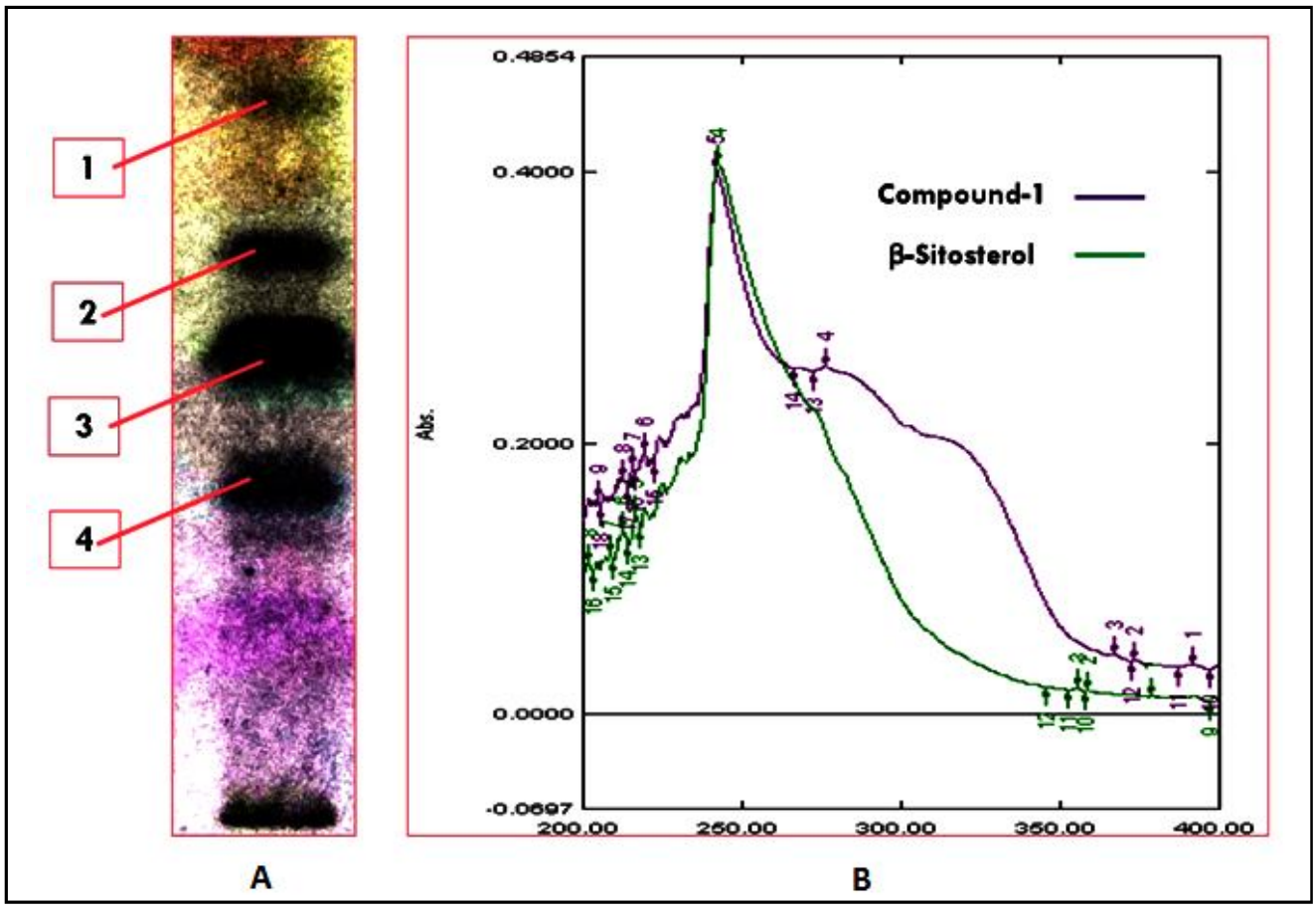

Figure 3 A: TLC of petroleum ether fraction, B:Overlay UV spectra of an isolated compound and standard BS.

3.2 Phytochemical screening

3.2.1 Preliminary tests

All phytochemical preliminary screening results are in Table 1 of EH tuber's MTE. Results meet the standard given to them.

Table 1: Preliminary tests for the isolated compound from EH's MTE

	Isolated compound to be identified
UV nm	242.80
IR cm^{-1}	3740.10, 2956.01=OH, 2316.58 = C-H =, 1455.34= C-H, 860.28 = C-H.
^{1}H NMR	δ 3.638 (1 H, m, H 3), 3.2(1H, m, H 3), 1.8 2.0 (5H, m), 5.26 (1H, m, H 6), 2.38(1 H, m, H 20), 5.19(1H, m, H 23), and 4.68 (1H, m, H 22) ppm. δ 1.35 1.6 (m, 9 H), 1.35 1.42 (m, 4 H), 0.76 0.89 (m, 9 H),1.07 1.13 (m, 3 H), 0.69 0.73 (m, 3 H), 0.91 1.05 (m, 5 H), 1.8 2.00 (m, 5 H) ppm
^{13}C NMR	79.03 (C 3), 55.3(C 4), 121.7, 118.89(C 6), 50.45 (C 9), 48.3 (C 9), 40.8 (C 20), 55.18(C 17), 40.1 (C 12), 38.9 (C 4), 38.6 (C 12), 339.2 (C 13), 7.12 (C 10), 36.3 (C 8), 32.66 (C 8), 26.1 (C 11,26), 35.59(C 20), 28.1 (C 15), 34.29 (C 22), 37.18 (C 1), 34.24 (C 7), 29.86 (C 25), 29.71 (C 16), 28.41 (C 2), 27.4 (C 28), 17.71 (C 21), 21.6 (C 27).

The absorption spectra studied in the 200-400 nm range show one absorption maxima for COMP-1 at 242.80 nm, shown in Figure 3. B and it is compared with the UV spectra of BS, whose absorption spectrum was found at 242.80 nm, shown in Figure 3 B. The FT-IR spectrum showed multiple absorption peaks in Table 2; the broad absorption peaks at 3740.10 and 2956.01cm^{-1} reported the presence of the -OH group. The stretching vibrations of aliphatic C-H were recorded at 2316.58 cm^{-1}. The significant peak at 1455.34 cm^{-1} and 860.28 cm^{-1} demonstrated the C-H stretch and bending vibrations, respectively. The FT-IR spectra of the isolated compound were identical to standard spectra of BS, as shown in Figure 4.

Table 2: IR ranges of MTE of EH tubers

S. No.	Absorption peak cm^{-1}	Functional group and pattern
1	3740.10	O-H group
2	2956.01	O-H stretching
3	2924.18	O-H stretch
4	2316.58	C-H streching vibration
5	1455.34	C-H stretch
6	860.28	C-H bend

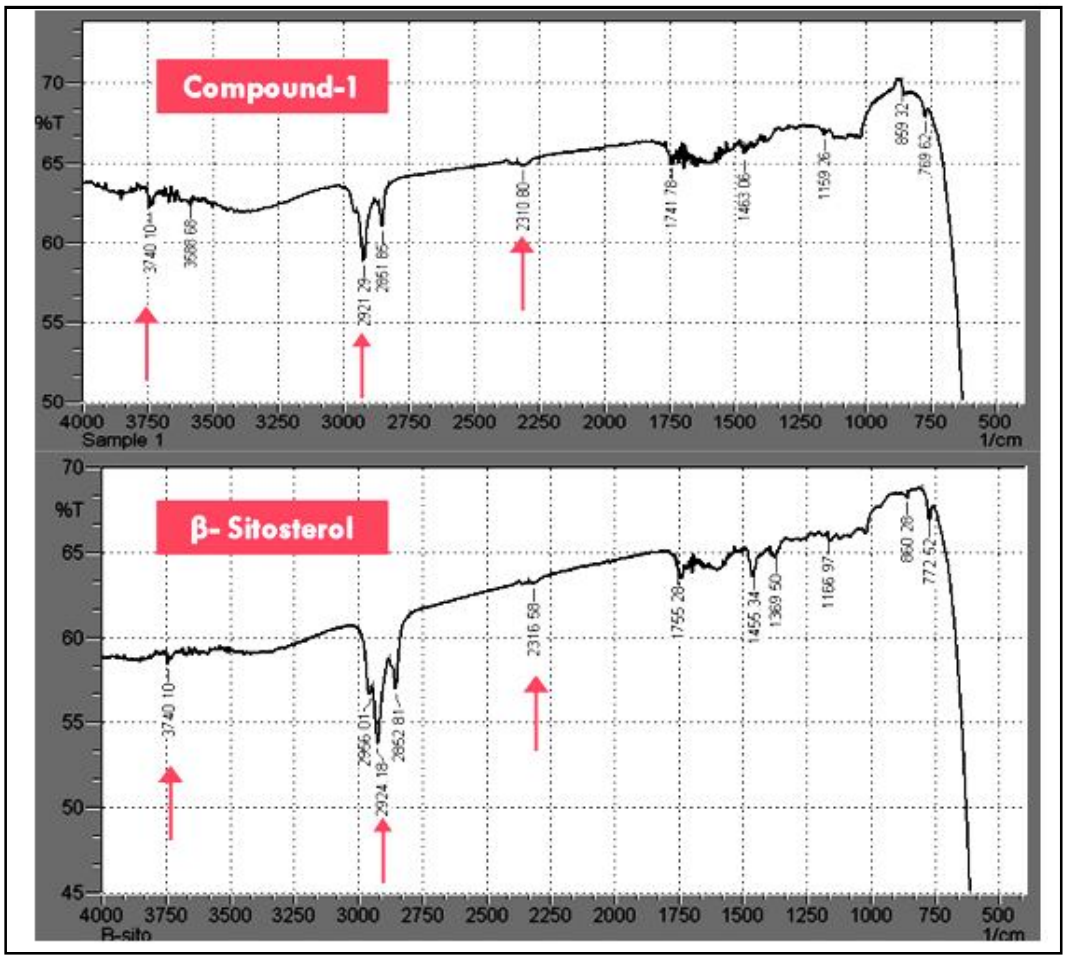

Figure 4: IR spectra of isolated COMP-1 and standard BS.

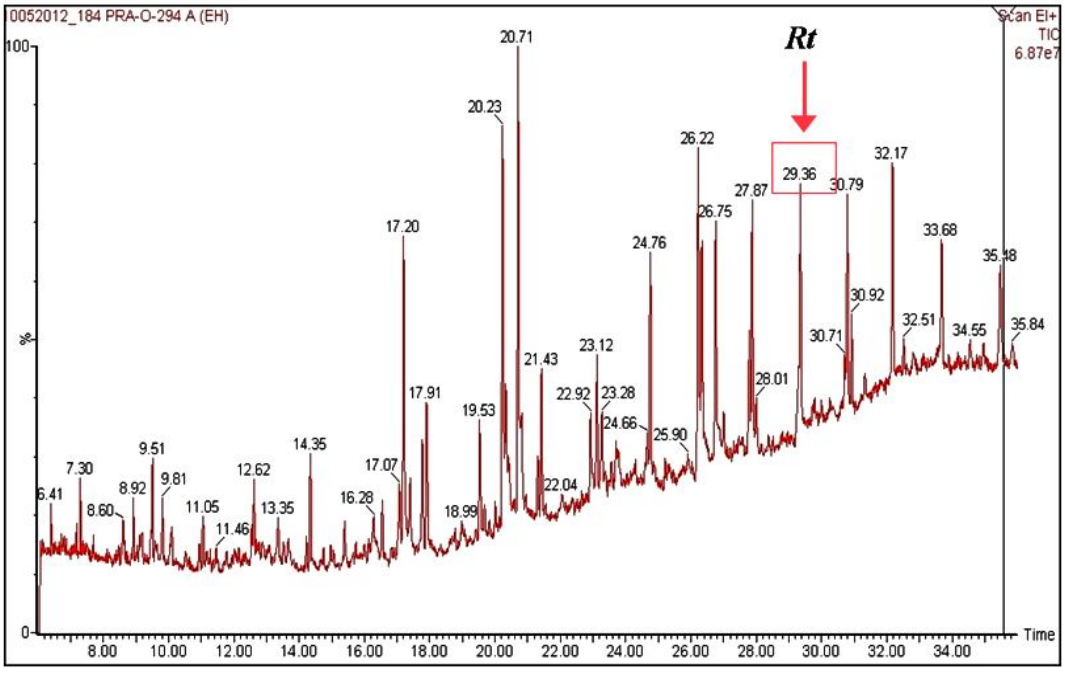

Figure 5: GC-MS of PEF.

The GC-MS/MS spectrum of COMP-1 was obtained from the column and analyzed qualitatively by GC-MS, as shown in Figures 5 and 6. The spectra disclosed the BS as a selected peak with Rt 29.36, representing the presence of m/z 414 and a perfectly matched fragmentation pattern compared with the standard. The fractionation FT-IR revealed the (OH stretching; 3373.6 cm ^{1}). The significant peak at 2867.9 cm ^{1} and 2940.7 cm ^{1} discovered the stretching vibrations of the C H aliphatic compound; 1641.6 cm ^{1} (C=C); other peaks such as 1381.6 cm ^{1}, 1457.3 cm ^{1}, 1038.7cm ^{1} demonstrated the presence of CH_2, OH, and cycloalkane as major functional groups within the fraction isolated.

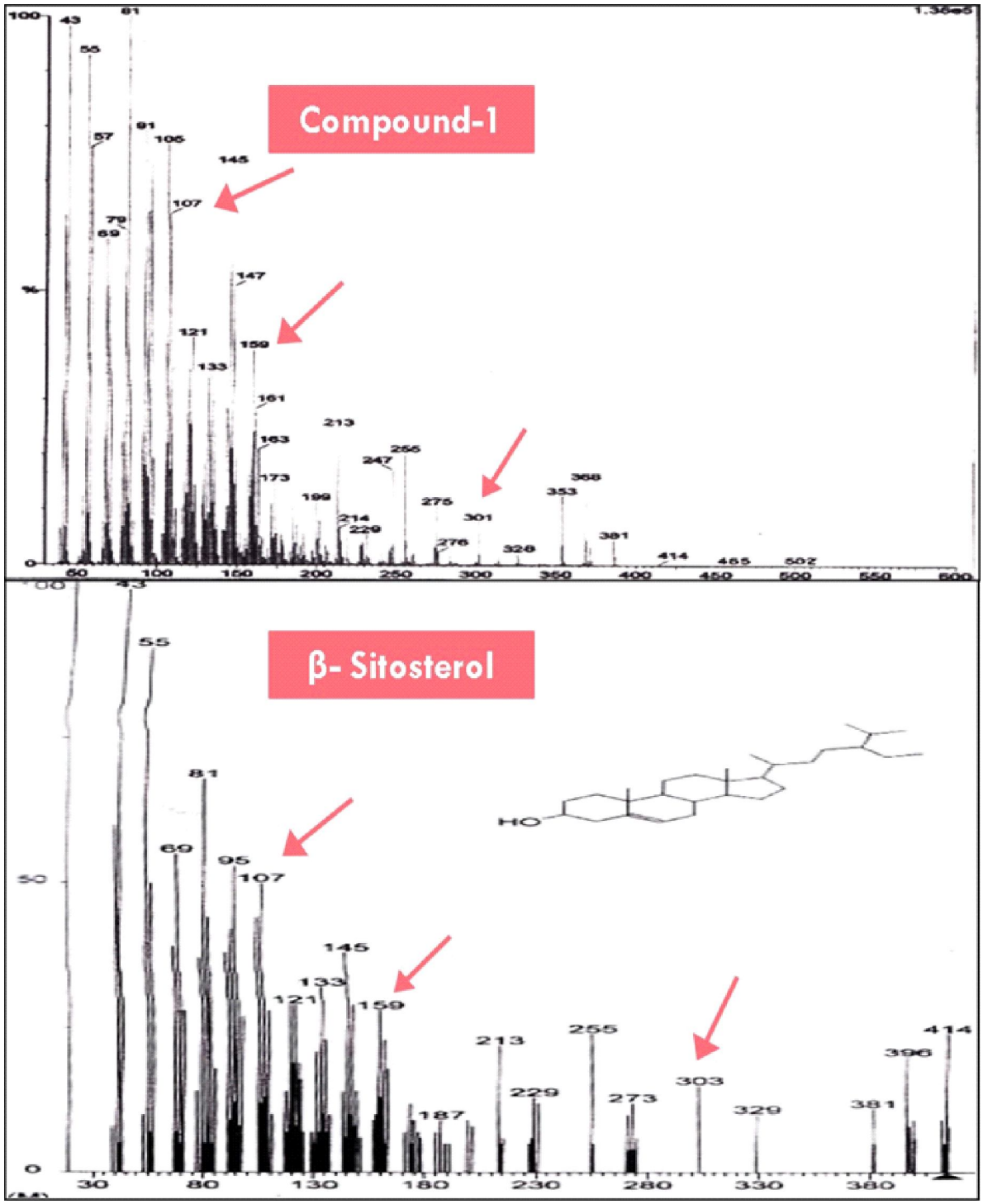

Figure 6: Mass spectrum of COMP-1/selected peak Rt (29.36) and standard BS.

The ^{1}H NMR spectra of COMP-1 displayed an H-3 proton appearance at δ 3.62 as a triplet of the double doublet with a J (6.5), and H-6 olefinic proton showed a multiple at δ 5.38. The isolated compound's ^{1}H NMR spectrum had a great agreement for the structure of BS, which are identical at δ 3.2(1_H, m, H_3), 5.19(1_H, m, H_23), 5.26 (1_H, m, H 6), 3.638(1_H, m, H_3), 1.8 2.0 (5_H, m), 2.38(1_H, m, H_20) ppm. Other peaks are observed at δ1.8 2.00 (m, 5H), , 0.91 1.05 (m, 5H), 1.35 1.42 (m, 4H), 1.35 1.6 (m, 9H), 0.69 0.73 (m, 3H), 0.76 0.89 (m, 9H), 1.07 1.13 (m, 3H) ppm these spectra are shown in Figure 7.The compound's ^{13}CNMR spectra showed the existence of 31 carbon molecules. The C5 carbon conjugation of alkene resulted from the chemical shifting at 150.98 ppm and 145.2 ppm. Similarly, the chemical shifts at _15.6 ppm and 19.32 ppm reflected the angular carbon C18 and C19. Other signals were received by 13CNMR at 121.7, 79.03 (C_3), 118.89 (C_6), 37.18 (C_1), 55.3 (C_4), 55.18 (C_17), 50.45 (C_9), 48.3 (C_9), 40.8 (C_20), 27.4 (C_28), 40.1 (C_12), 39.2 (C_13), 26.1 (C_11,26), 38.6 (C_12), 37.12 (C_10), 34.24 (C7), 32.66(C_8),35.59 (C_20), 29.71 (C_16),28.1(C_15), 28.41(C_2),17.71 (C_21). The values of isolated compounds in the ^{13}CNMR spectrum were identical to the standard BS Figure 7.

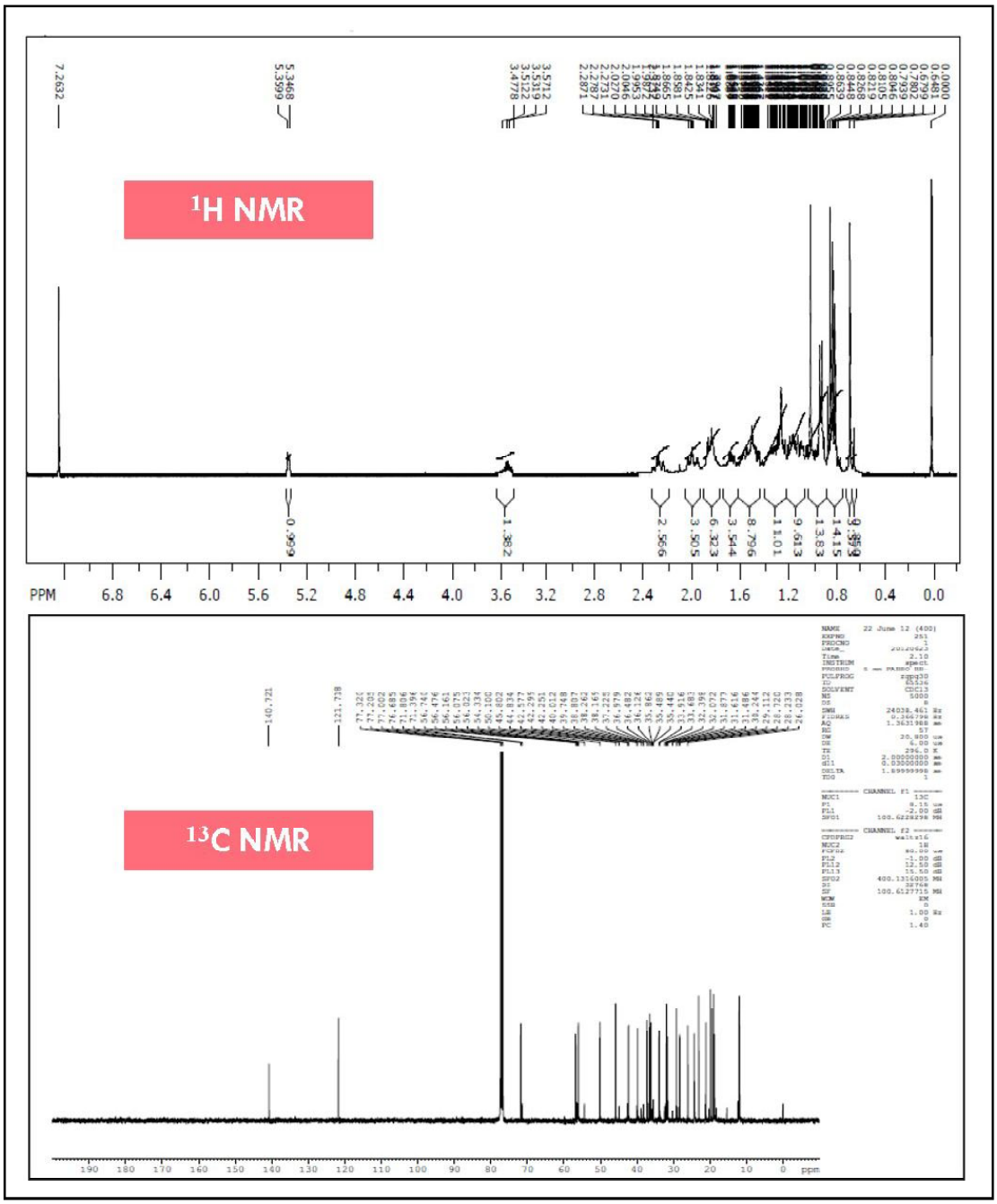

Figure 7: ^{1}H NMR and ^{13}C NMR spectrum of COMP-1.

The spots of the COMP-1 and standard BS had the same color and Rf values on TLC plates after being detected by visualizing reagents and TLC plats showed the approximate difference between COMP-1 and standard BS in Rf because of the similarity in structure for the 2 compounds. EH's MTE underwent a preliminary screening that revealed the presence of significant phytoconstituents such as carbohydrates, mucilage, amino acids, tannins, steroids and triterpenoids. Depending upon the above results and analysis, the isolated compound found to be BS is confirmed and forwarded for the cell line study discussed below.

3.3 Anticancer activity using SRB assay

Anticancer efficiency of methanol extract of EH tuber was screened by using CC cell line (205) at concentrations of 10-80 µg/ml as indicated in Tables 3 and 4.

Table 3: % control growth of cell lines in the presence of EH tuber's MTE and standard adriamycin

% control growth				
Concentrations (µg/ml)	**10**	**20**	**40**	**80**
MTE*				
Experiment 1	67.1	68.8	81.0	119.8
Experiment 2	64.1	70.2	79.9	97.7
Experiment 3	73.0	77.2	79.5	107.6
Average values	68.1	72.1	80.1	108.4
EAF*				
Experiment 1	65.1	67.8	84.2	82.0
Experiment 2	65.4	71.3	66.8	90.7
Experiment 3	73.4	73.7	65.9	75.0
Average values	67.9	70.9	72.3	82.6
COMP-1*				
Experiment 1	59.5	48.1	36.0	29.6
Experiment 2	57.0	48.8	31.4	37.0
Experiment 3	62.8	55.0	30.7	28.8
Average values	59.8	50.6	32.7	31.8
ADR*				
Experiment 1	-1.0	-10.5	7.5	13.4
Experiment 2	4.5	3.1	8.6	15.9
Experiment 3	3.9	-1.5	5.3	16.7
Average values	2.5	-3.0	7.1	15.4

*ADR = Adriamycin (Positive control compound), *COMP-1= Compound one (Isolated BS)
*EAF=Ethyl acetate fraction, *MTE= Methanolic extract.

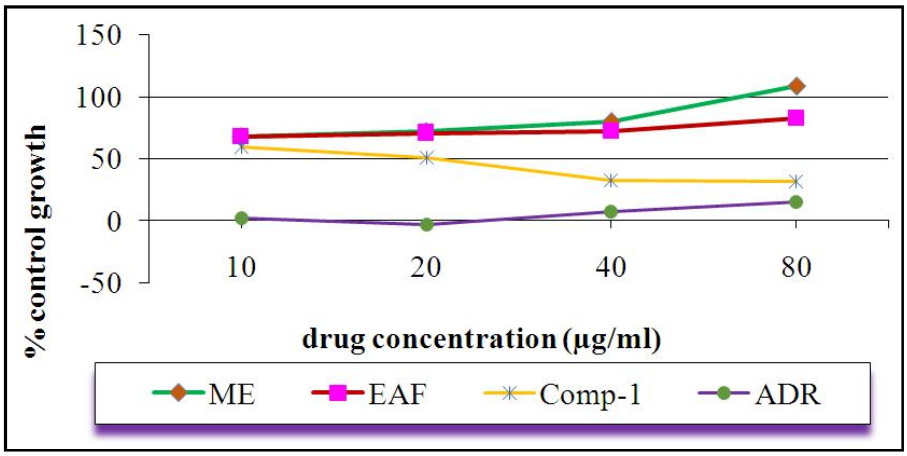

Figure 8: % control growth vs drug concentration.

Table 4: Lethal concentration and other values of drug concentrations from the graph

Name of drug [µg/ml]	LC_{50}	TGI	GI_{50}
Methanolic extract (MTE)	NE	>80	NE
Ethyl acetate fraction (EAF)	NE	>80	NE
COPM-1-Isolated BS	NE	>80	21.0
Adriamycin	NE	13.4	<10

LC_{50} = Conc. of the drug causing 50% cell kill

GI_{50} = Conc. of the drug causing 50% cell growth inhibition

TGI = Conc. of the drug leading to total cell growth inhibition

NE = non-evaluable data

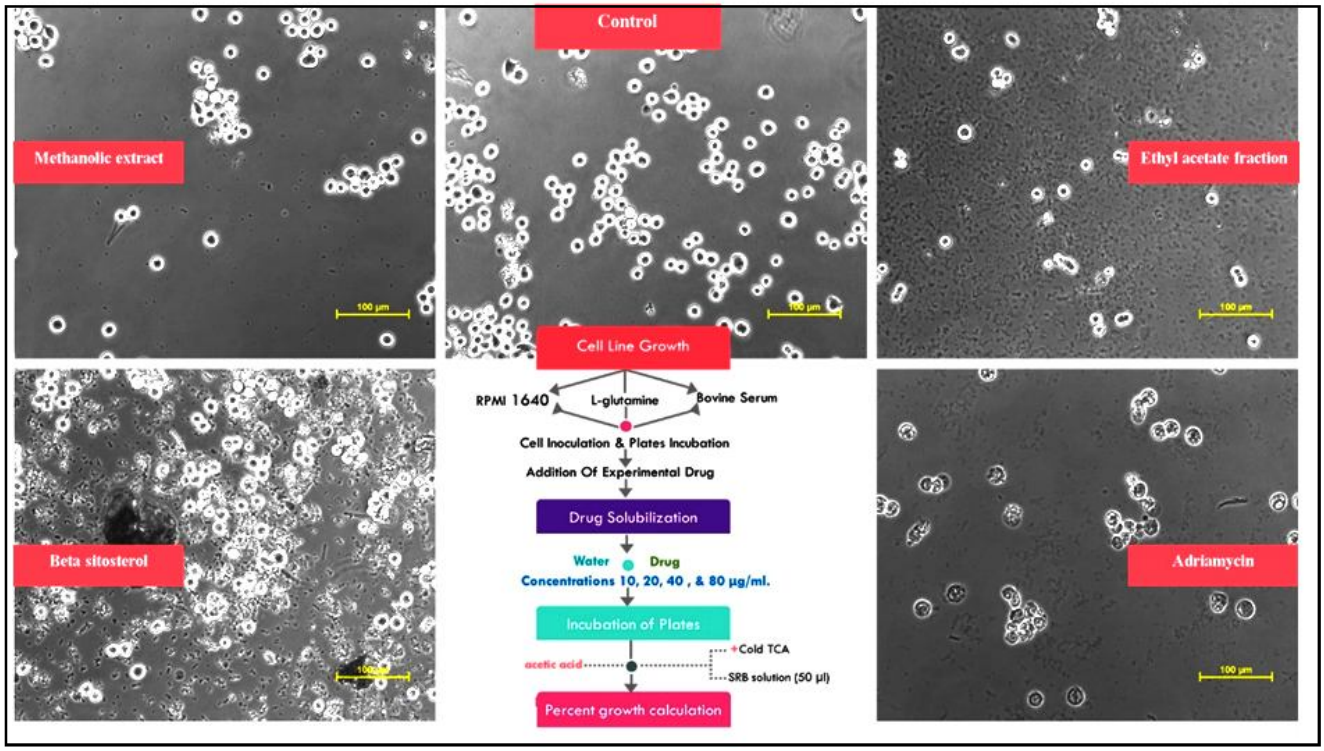

Figure 9: Colo-205-cell line growth.

The extract showed the % growth of inhibition for the COLO 205 in a concentration-dependent manner (Figures 8 and 9). Its effect was found to be more significant in the human CC cell line (205) at 80 µg/ml, while the minimum at 10 µg/ml. The cell line's LC_{50} and TGI were > 80 µg/ml. The concentration of GI_{50} for COMP-1 was < 21.0 µg/ml against the cell line, revealing the anticancer effectiveness of MTE.

Several earlier investigations disclosed that herbal extract possesses plenty of phytoconstituents with antineoplastic attributes and might be accountable for antineoplastic action. Plants of the Orchidaceae family are an abundant source of BS, an anticancer Phytoconstituents. The anticancer properties of the MTE of EH may be attributed to the presence of BS.

4. Discussion

Present investigation was designed to generate phytochemical and pharmacological screening of herbal plant tuber, *E. herbacea*. The study involved extraction and chemoprofiling of *E. herbacea* to assess their anticancer activity. As we all know, there are hazardous side effects of synthetic drugs as compare to herbal drugs, hence there is a need to explore such new medicinal plants having therapeutic values. The methanolic extract showed the presence of secondary metabolites that was confirmed by phytochemical screening; then isolation done by column and preparative TLC further proceed for the UV IR NMR mass analysis confirmed that the isolated compound is β-sitosterol. The β-sitosterol active against some cancer cell like 549 and have cytotoxic potential (Prabhakar *et al.*, 2014; Raiavel, 2018). β-sitosterol affords many important health aids. It reduces the level of LDL and lower the risk of coronary artery disease, heart attack. It is also helpful in preventing many types of cancers and support for recovery of body. β-sitosterol had chemopreventive potential and it is less harmful to normal cells (Lkovaâ *et al.*, 2004). The anticancer activity of isolated compound

and extract was studied by SRB assay on human colon cancer cell line (Colo 205). In SRB assay, it was found that all compounds show dose dependant activity against cancer cell lines.The extract suppresses the cell line's per cent control growth in a dose-dependent way. In a pharmacological investigation, compounds were shown to have anticancer action against human cancer cell lines.

5. Conclusion

In the era of 21st century, cancer disorders are considered as major risk factors. It has been believed that this will turn out to be the main cause of death and disability worldwide by the year 2030, therefore, it should be treated effectively. The current target based synthetic drugs available for treatment are not fulfilling need of safe and long-term therapy to bring the balance back to normal conditions.The tubers of EH contain BS that has been proven to exhibit significant anticancer potential against CC cell line (205) and can be considered for further development as biomedicine. Further, more definite studies are obligatory to demystify BS activities that would assist certain terminally ill patients, particularly those suffering from breast and CC.

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Authors contribution statement

Dr. Anil Tatiya designed and conceptualized this research work, Mrs. Snehal Bhavsar collected the sample analysed, calculated the data and interpreted all the data. Both authors contributed in revising and editing the manuscript.

Conflicts of interest

The authors declare no conflicts of interest relevant to this article.

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