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Original Article

IN-VITRO STUDY OF CO_2 EXTRACT OF *TERMINALIA CHEBULA* IN BREAST CANCER CELL LINE MD-MB-231

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ABSTRACT

Cancer is an abnormal growth of cells in body which leads to death. These cells are born due to imbalance in cell proliferation mechanism. In 2018, WHO released new statistics on cancer incidence, mortality, and prevalence worldwide i.e., GLOBOCAN 2018 estimates for 28 types of cancer in which more prevalence of cervix and breast cancer. According to survey, in India about 7.8 million cancer deaths and 11.5 million new cases arise in 2018, which will increase to 19.3 million new cases per year by 2025. Though breast cancer as such is not explained anywhere in Ayurvedic compendia, correlations can be done with the *Stana Arbuda*. *Ayurveda*, the ancient system of medicine came into existence 1000's of years ago with an objective of maintaining the health of people and treating diseases. Many herbs used in *Ayurveda* have been screened for activity against cancer and in-vitro and in-vivo studies have given promising leads. The plant, called as "Mother of Medicine", *Haritaki* has been extensively studied for its various ailments because of its extraordinary healing potency. *Haritaki* (*Terminalia chebula* Retz.), Family: Combretaceae have a great therapeutic value and is widely distributed in India. Dried fruit of *Terminalia chebula* contains high quantities phenolic compounds consist of ellagic acid, gallic acid and chebulic acid. The fruit extract of *T. chebula* is having different biological properties like anticancer, antioxidant, hepatic and renal protective activities etc. In this study, we focus on the use of CO_2 extract of *Terminalia chebula*, on the breast cancer cell line MDA-MB-231. All tests proved that CO_2 extract of *Terminalia chebula* containing active chemical component, therefore our experiment showed the positive results for CO_2 extract of *Terminalia chebula* against breast cancer cell line cancer MDA-MB-231. The MTT assay results were used to evaluate the anti-cancer activity of the extract. The percentage of cell growth and cell viability were calculated from tabulated result values of MTT assay. Cell viability MTT assay also showed significant growth inhibition, at the same time statistical analysis of MTT assay also proved significant results.

Keywords *Haritaki*, *Terminalia chebula*, CO_2 extract.

1. INTRODUCTION

Cancer is one of the most dreadful disease of present century. It is leading cause of mortality, it is the cause of more than 20% of all deaths. According to survey 8.2 million cancer deaths and 14.1 million new cases arise in 2012, which will increase to 19.3 million new cases per year by 2025. It is an abnormal growth of cells in body which leads to death. These cells are born due to imbalance in cell proliferation mechanism. Cancer begins with mutations in DNA, which instructs the cells how to grow and divide. Breast cancer is most frequent cancer in women responsible for almost 20% of all cancer deaths. It is leading killer among women aged 20-59 years. Its mortality have increased over the past 30 years. Breast cancer is most common worldwide, with nearly 1.7 million new cases diagnosed and 521,900 deaths in 2012. (Globocan.iarc.fc/old/factsheets.18.01.2015)

The description of "*granthi*" and "*arbuda*" by *Charaka* and *Sushruta* can be interpreted as cancer growth in the body. Imbalance of three *doshas vata*, *pitta* and *kapha* in body are responsible for disease. Though breast cancer as such is not explained anywhere in Ayurvedic compendia, correlations can be done with the *Stana Arbuda*. However, *Stana Arbuda* is not mentioned directly in Ayurveda, we can correlate references about it in Ayurvedic texts like *Sushruta Samhita*, *Ashtanga Hridya* and *Madhava Nidana*. There are different kinds of

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pathologies explained which are quite similar to cancer; like, *Granthi, Arbuda, Apachi* etc. among these, the signs and symptoms of *Arbuda* fits more into the definition. Several herbs used in Ayurveda have been screened for activity against cancer and in-vitro and in-vivo studies shown encouraging leads. Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological and medicinal activities, higher safety margins and lesser costs.

Haritaki (Latin name - *Terminalia chebula*, family - Combretaceae). *Haritaki* is commonly termed as 'King of Medicine' due to its promising medicinal value in the management of various diseases. *T. chebula* is native plant of India and its dried fruits have many medicinal properties. *Terminalia chebula* is a flowering evergreen tree of the family *Combretaceae*. It has several common names like black *myrobalan*, ink tree, or chebulic myrobalan (English), *Harad* (Hindi), *Haritaki* (Sanskrit and Bengali), *Harada* (Marathi and Gujrati). It well known as '*Haritaki'* since it carries away all diseases or it is sacred to God Shiva (Hara). *Haritaki* has several interesting synonyms like '*Pathya'*, since it removes obstructions from the pathways and channels in the body; '*Abhaya'*, since it gives fearlessness; '*Amrta'*, means an ambrosia; '*Divya'*, means a divine herb; '*Medhya'*, means a nerve tonic; '*Pranada'*, means lifesaving; '*Jivaniya'*, means a vitalizing herb; '*Vayahstha'*, means one that promotes longevity and maintains youth, etc. [*Nighantu Adarsha*] In Indian mythology, this plant is supposed to be originated from the drops of Ambrosa (Amrita) which fell on the earth when God Indra drunk it. Furthermore, the plant has been well reported to possess antioxidant, anti-diabetic, antibacterial, antiviral, antifungal, anticancer, antiulcer, anti-mutagenic, wound healing activities (Suchalatha S *et al.,* 2005; Rao NK *et al.,* 2006; Kannan P *et al.,* 2009). *Haritaki* contains chebulinic acid, tannic and ellagic acid, which are found to be the most growth inhibitory phenolics (Saleem A *et al.,* 2002). Chebulagic acid is the compound in *Haritaki* which is responsible for anti-inflammatory activity (Reddy DB, 2009). *Haritaki* shows that tri-ethyl chebulate is a strong anti-oxidant (Xiupingchen *et al.,* 2011). It is used in traditional medicine due to wide spectrum of pharmacological activities. Because of these enormous medicinal properties, *Haritaki* can be called a 'wonder herb'. In *bhavaprakash nighantu, Haritaki* is described as *deepana, pachana, rasayana, tridosha shamaka* and *balya*. According to *Acharya Sushruta* and *Vagabhatta, Haritaki* powder is used in *Pittaj granthi*. (*Sushrut chikitsasthan* 18/8, *Ashtang Sangrah Uttarsthan* 35/8)

The fruit of *Haritaki* contains five *Rasas* namely:

i. *Madhur* (sweet) - the fruit pulp,
ii. *Amla* (sour) - the bulky portion of the fruit,
iii. *Tikta* (bitter) – seed,
iv. *Katu* - the covering of the fruit and
v. *Kashaya* (astringent) - the hard portion of the seed.

Thus, *Haritaki* is *Pancharasatamak.* [*Bhavaprakash Nighantu*]

Properties and Action

Rasa: Madhura, Amla, Katu, Tikta, Kashaya

Guna: Laghu, Ruksha

Virya: Ushna

Vipaka: Madhura [Dravyaguna Vijyana, Vol.- II page-753]

Karma: It acts as *Tridosha Shamaka* (mitigates *Vata, Pitta* and *Kapha*). *Shothahara* (anti- inflammatory), *Chakshushya* (beneficial for eyes), *Mukharogahara* (cures oral diseases), *Balya* (strengthening), *Deepana* (appetizer), *Paachana* (digestant), *Vatanulomana* (carminative), *Vruna Ropana* (wound healing), *Yakrita-Pleeha Uttejaka* (hepatoprotective), *Kasa -Swasahara* (cures cough and breathlessness), *Hikka* (cures hiccough), *Mootrala* (act as diuretic), *Rasayana* (rejuvenator), *Vajikara* (aphrodisiac). [Dravyaguna Vijyana, Vol.- II page-753]

Therapeutic Uses - *Shotha, Arsha, Aruchi, Hrydroga, Kasa, Pandu, Prameha, Udhvarta, Vibandha, Vishamajvara, Shiroroga, Tamaka Swasa, Gulma, Udararoga*. [Dravyaguna Vijyana, Vol.- II page-753]

Useful part: Fruit, Root and Bark

Important Formulations -*Triphala Choorna, TriphaladiTaila, Abhayarishta, Agastya Haritaki Rasayana, Chitraka Haritaki, Danti Haritaki, Dashmul Haritaki, Bramha Rasayana, Abhaya Lavan, Pathyadi Lepa.* [Dravyaguna Vijyana, Vol.- II page-753]

Dose– Fruit Powder – 2-6 gm Bark Powder – 1-4 gm *Kwath* - 25 – 50 gm. *Arishta* – 2 ½ Tola (30ml) [Raj Nighantu]

Traditional values of *Haritaki* – In *Charaka Samhita* and *Sushruta Samhita*, various medicinal plants has been mentioned but *Haritaki* is placed in prime among medicinal plants. It is a top listed plant in Ayurvedic Materia medica for treatment of asthma, bleeding piles, sore throat, vomiting and gout (Aneja KR *et al.,* 2009). It is used in Thai traditional medicine as a carminative, astringent and expectorant (Panunto W *et al.,* 2011). According to *Vagbhata*, it is the drug of choice in the therapy of '*Vata-Kapha'* diseases. The '*Triphala'*, a herbal formulation of 'three fruits' from plants *Terminalia chebula, Terminalia Bellerica, Emblica officinalis*, are used as laxative in chronic constipation, food digestive problems (poor digestion and assimilation) detoxifying agent of the colon, and rejuvenator of the body (Prasad L *et al.,* 2006). The fruits of *Haritaki* are used both externally as well as internally for medicinal purposes. Externally, the paste of fruits effectively reduces the swelling, hastens the healing and cleanses the wounds and ulcers. In erysipelas and other skin disorders, *Haritaki* prevents accumulation of pus in skin diseases. The oil of *Haritaki* is extremely helpful in healing of wounds especially in burns. The paste of fruit is also applied in conjunctivitis for relief due to its anti-inflammatory property. The gargles with its decoction give excellent results in stomatitis and problems of the throat. A fine powder of *Haritaki* is used as a tooth powder to strengthen the gums. Internally, *Haritaki* is used to cure a vast of disease. According to *Vagabhatta*, when *Haritaki* powder fried in ghee is regularly consumed with sufficient ghee in food, it promotes longevity and boosts energy. Common gastrointestinal ailments, tumours, ascites, piles, enlargement of liver and spleen, worms, colitis can be treated well with *Haritaki.* '*Bala Haritaki'* is useful in haemorrhoids and in clearing the bowels. In abdominal pain due to flatulence, it is given with jiggery and ghee. The most popular combination of *Haritaki, Musta, Shunthi* and jaggery is an effective panacea for diarrhoea, dysentery, and flatulence etc. '*Haritaki Siddha Ghrita'* is beneficial in chronic fever. *Haritaki* powder with honey and ghee is also effective remedy for anaemia. In obesity, its decoction with honey reduces the excessive body fats. Regular use of *Haritaki* improves memory due to beneficial effects on the nerves of brain. It is also valuable in dysuria and urinary stones. (Kirtikar KR *et al.,* 1935)

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AIM OF THE STUDY

The present study was done to evaluate the use of '*Haritaki' Terminalia chebula* Retz. as an anti-cancer drug in breast cancer by in-vitro study.

MATERIALS AND METHODS

Plant Material

Fruits of *Haritaki* (*Terminalia chebula* Retz.) were procured from Organic Farm in Umbari west of Satara District in Mahararshtra.

Authentication and voucher deposition – It was done in the Botany Department of authorised Institute. A voucher specimen of the collected sample was deposited in the departmental museum for future reference.
Accession No. – HC0024

Preparation of CO_2 Extract of *Terminalia chebula* (S. Sasidharan *et al.*, 2011)

1. Material – Powdered form of fruit of *Terminalia chebula*. Choorna was prepared according to the guideline mentioned in *Sharangdhar Samhita* and subjected to standardization.
2. Equipments –
    - A. CO_2 Tank
    - B. Pump
    - C. R/M input
    - D. PRV
    - E. Cyclone Separator
    - F. Collection vessel.

Preparation of CO_2 Extract

First of all CO_2 which was stored in tank is pumped to the extractor (Fig. 2) where pressure was maintained at 300 bar (=296.077atm) and at the temperature 304 K (31°C). In the extractor, liquid CO_2 and feed (Fig. 1) come in contact with each other and extraction of essential component from feed was carried out. The product steam which contains CO_2 and essential component goes to the cyclone separator through PRV (Pressure relieving valve), due to deduction in pressure PRV liquid CO_2 is converted into gas. In cyclone separator CO_2 gas and essential component (liquid form) are separated from each other. From the top of the cyclone separator CO_2 is recycled back to the tank, before sending it to the tank, CO_2 which turned to gaseous state is condensed with the help of condenser. From the bottom of the cyclone separator essential components i.e. CO_2 extract of *T. chebula* (Fig. 3) was obtained.

Fig.1 Core Powder of *T. chebula*

Fig.2 CO_2 Extractor Machine

Fig.3 CO_2 extract of *T. chebula*

Cell Line Culturing

Cell line – MDA-MB-231 Breast Cancer Cell Line
Source – APT Research Foundation, Pune (Maharashtra)
Procedure -Hands was sprayed with 70% ethanol. Removed T-flasks from the incubator which contains cell lines. Checked cells in flask under microscope to confirm that the cells are 90% confluent. Old media/ spent media from flask was removed. Phosphate buffer saline (PBS) for eg.2 ml in T25 flask likewise 4ml/6ml PBS was added in T75/T225 flask respectively and shook thoroughly to detach all the cells. Within few second / immediately remove PBS or Pipette out from flask. Then trypsin was added for e.g. 1 ml in T25 flask likewise 2ml/4ml in flasks T75/T225 respectively. Then flask containing trypsin and cell lines were kept in incubator for 3 minutes (if kept more than 3 min cells will get stressed). After 3 min took the flasks from incubator and made sure that all the cells were suspended in the trypsin by gently tapping. Then added 5 ml of complete media in T25 flasks. For e.g. 15ml in T75 and 25 ml in T225 flasks. Then pipettes out in centrifuge tube or falcon tube. This was also simultaneously taken for cell counting and the result was noted. Centrifuge for 2-3 minutes the pellet is formed then took pellet and discard supernatant. Fresh media was added to the falcon tube and was pipettes in and pipettes out several times dislotch. For e.g. 5ml in T25 flask. For equal distribution of cells the flask was moved in infinity symbol pattern (). It was sealed by parafilm to prevent contamination. Labelled the flask by cell line, passaging number. The flask was kept in the incubator for 24 hrs. (Fig. 4)

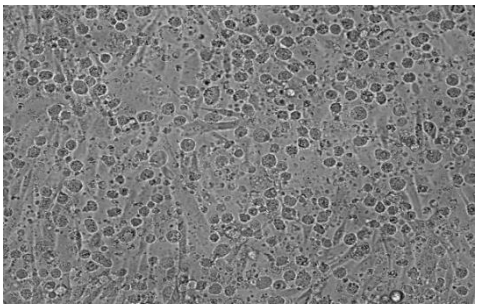

Fig.4 Cultured MDA-MB-231 cell line

Phytochemical analysis

CO_2 extract of *Terminalia chebula* was screened for the presence of various Phytoconstituents using standard procedures. In CO_2 extract of fruit of *Terminalia chebula*

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following constituent like Tannin, Alkaloid, Phenol, Carbohydrate, Steroids, Protein, Resin were detected.

HPTLC-

HPTLC fingerprint of CO_2 extract of fruit of *Terminalia chebula* is little bit similar as of phytochemical analysis. The results indicate that *Terminalia chebula* contains a number of markers that may be responsible for its anticancer activity. The developed HPTLC method will assist in the standardization of *Terminalia chebula* using biologically active chemical markers. The proposed Validated HPTLC method for Gallic acid from *Terminalia chebula* seems to be accurate, precise, reproducible and repeatable. *Terminalia chebula* also contained a number of other constitute, which was shown in phytochemical analysis. Hence, the present study has been helped to given out the parameters to determine the anticancer activity of CO_2 extract of fruit of *Terminalia chebula*. When HPTLC study of CO_2 extract of *Terminalia chebula* was carried out with the help of Mobile phase Toluene: Ethyl acetate: Formic acid (3:6:1v/v) for Gallic acid. Prominent light bands detected at R_f value is 0.41 under at 280nm. The method was found to be very specific. Since the overlay spectrums of standard Gallic acid with sample of *Terminalia chebula* were found to be similar. Quantification of Markers present in *Terminalia chebula* was 0.9689% w/w.

Cell Viability MTT Assay –

Day 1 - 104 cells/well was added in a 96 well tissue culture treated plate (Cell count was taken on a Neubauer's chamber). The plate was incubated at 37°C in a 5% CO_2 incubator for 24 hours.

Day 2 - After 24 hrs incubation, the plate was observed under inverted microscope to check the morphology of the cells and confluency of the wells in the 96 well plate. Sterile test sample was suspended in DMEM containing 10% FBS at a known concentration and dilutions for the same were made accordingly. 100 µl of each of the test samples were added in triplicates along with the positive control (DMSO) and normal control (cells with medium and no test sample). Post sample addition, the plate was incubated at 37°C in a 5% CO_2 incubator for 24 hours.

Day 3 - After 24 hours incubation, the plate was observed under the inverted microscope and photographs were taken of the recorded observations. Test sample was removed and 90 µl fresh DMEM containing 10% FBS was added. Then 10µl of MTT reagent was added in each well. The plate was wrapped in aluminium foil and incubated at 37°C in a 5% CO_2 incubator for 4 hours. Post 4 hours incubation, the entire medium was removed by flicking the plate and 100 µl of solubilisation buffer was added in each well and incubated at 37°C in a 5% CO_2 incubator for about 20 minutes. Post incubation, absorbance was measured at 630 nm on 96 well Plate reader.

RESULTS AND DISCUSSION

Preparation of CO_2 extract of *Terminalia chebula*
Solvent: CO_2 in liquid form
Herb (powder of *Terminalia chebula* fruit) to Extract Ratio = 165:1
% yield of extract = 0.60 %
Phytochemical analysis
While studying the phytochemical analysis we have found constituent like Tannin, Alkaloid, Phenol, Carbohydrate, Steroids, Protein, Resin in CO_2 extract of fruit of *Terminalia chebula*. (Table 1)

Table 1. Phytochemical screening of CO_2 extract of *Terminalia chebula*

Sr.No.	Test for	Result
1.	Saponin test	Negative
	Foam test	
2.	Tannin test	Positive
	Ferric chloride test	
3.	Alkaloids test	Positive
	Wagner's test	
	Mayer's test	
	Hager's test	
4.	Glycoside test	Negative
	Legal test	
5.	Phenol test	Positive
	Ferric chloride test	
6.	Carbohydrate test	Positive
	Fehling's test	
	Benedict's test	Negative
7.	Steroids test	Positive
	Salkowski test	
8.	Proteins test	Positive
	Biuret's test	
9.	Carboxylic acid test	Negative
	Sodium bicarbonate test	
10.	Coumarins test	Negative
	Sodium hydroxide test	
11.	Resin test	Positive
12.	Quinine test	Negative

Cell Line Observation (Table 2)

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Table 2. Stastistical Evaluation of Effect of CO_2 Extract of *Terminalia chebula* on Breast cancer MDA-MB-231 Cell line

Concentration ug/ml	1	2	3	Average
C	0.285	0.281	0.292	0.286
PC	0.228	0.218	0.227	0.224
50 ug/ml	0.281	0.289	0.275	0.282
125 ug/ml	0.259	0.265	0.263	0.262
250 ug/ml	0.248	0.242	0.235	0.242
500 ug/ml	0.189	0.179	0.171	0.180
1000 ug/ml	0.134	0.123	0.130	0.129

ANOVA test was applied to prove the treatment statistically significant or not, as there were 7 groups for anticancer activity CO_2 extract of *Haritaki* (*Terminalia chebula* Retz.) in MDA-MB-231 breast cancer cell line. As per table 2 the test drug i.e. CO_2 extract of *Haritaki* (*Terminalia chebula* Retz.) the mean percentage observed for 50µ/ml was 0.28167 with standard deviation of 0.007024, Mean percentage observed for 125 µ/ml was 0.26233 with standard deviation of 0.003055, Mean percentage observed for 250 µ/ml was 0.24167 with standard deviation of 0.006506, Mean percentage observed for 500 µ/ml was 0.17967 with standard deviation of 0.009018 Mean percentage observed for 1000 µ/ml was 0.12900 with standard deviation of 0.005568. For comparison among these groups, we have used One Way ANOVA test. From above table it was observe that P Value is less than 0.001.

Cell Viability MTT Assay

The MTT assay was used for in vitro experiment to determine the effect of *Terminalia chebula* Retz. Extract on breast cancer cell line MDA-MB-231.has been carried out at the department of *Ilmul Advia* in Regional Research Institute of Unani Medicine (RRIUM), and University of Kashmir-Srinagar. The aerial parts of the plants were studied for their pharmacognostic standardization and anxiolytic activity.

EFFECT ON MDA-MB-231 BREAST CANCER CELL LINE – (Fig. 2)

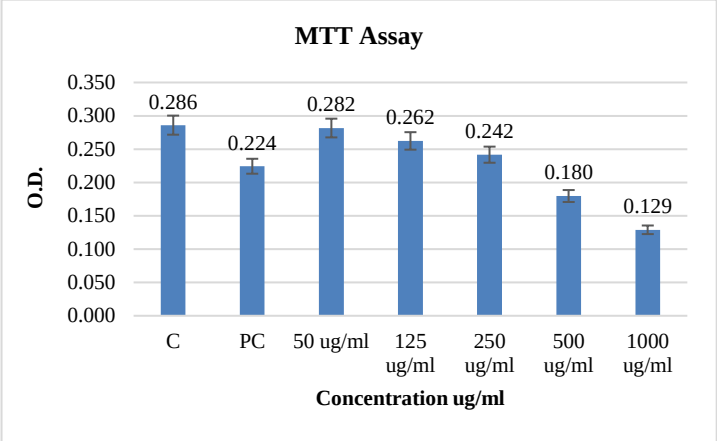

Fig.2 Graphical representation of Data obtained from invitro-study using MDA-MB-231 Cell line and CO_2 extract of *Terminalia chebula* fruit with respect to Control Growth and Drug Concentration

As per result obtained from MDA-MB-231 breast cancer cell line, the concentration of the study was increased upto 1000µg/ml. DMSO was used as Positive control group. Bar diagram is plotted on the basis of average value obtained from each experiment which denotes the effect of drug and control drug on selected cancer cell line.

The results have been discussed of anticancer study on breast cancer cell line MDA-MB-231 were as follows:

As compared to Control, Optical Density of Positive Control was significantly ($P<0.001$) less indicating significant cell death. Test sample has shown statistically significant increase in the Optical Density at 50 µg/ml ($P<0.001$) and 125 µg/ml ($P<0.001$) as compared to Positive Control indicating less toxicity at respective concentrations. At 250 µg/ml concentration, test sample has not shown statistically significant difference as compared to PC. At 500 µg/ml ($P<0.001$) and 1000 µg/ml ($P<0.001$) the Optical Density was found to be significantly decreased as compared to PC. Therefore overall data suggested that in comparison with PC; the test sample is less cytotoxic at 50 µg/ml and 125 µg/ml concentrations.

Statistical analysis

ANOVA test was applied to prove the treatment statistically significant or not, as there were 7 groups for anticancer activity CO_2 extract of *Terminalia chebula* Retz. in MDA-MB-231 breast cancer cell line. (Table no.3 and Table no.4)

Table 3. Calculation of P-Value for MDA-MB-231 Cell line at Different Concentration.

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Descriptives								
OD								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
C	3	0.28600	0.005568	.003215	.27217	.29983	.281	.292
PC	3	0.22433	0.005508	.003180	.21065	.23801	.218	.228
C50	3	0.28167	0.007024	.004055	.26422	.29911	.275	.289
C125	3	0.26233	0.003055	.001764	.25474	.26992	.259	.265
C250	3	0.24167	0.006506	.003756	.22550	.25783	.235	.248
C500	3	0.17967	0.009018	.005207	.15726	.20207	.171	.189
C1000	3	0.12900	0.005568	.003215	.11517	.14283	.123	.134
Total	21	0.22924	0.054680	.011932	.20435	.25413	.123	.292

Table 4. Effect on MDA-MB-231 Breast Cancer Cell Line

ANOVA					
OD					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.059	6	.010	251.662	0.000
Within Groups	.001	14	.000		
Total	.060	20			

The mean difference is significant at the 0.05 level. It indicates that CO_2 extract of *Terminalia chebula* able to show positive results on MDA-MB-231 Cell line as compare to Standard drug DMSO. Hence, from the above discussion it can be stated that CO_2 extract of *Terminalia chebula* shows anticancer activity.

CONCLUSION

The CO_2 extract of *Terminalia chebula* Retz. showed presence of Tannin, Alkaloid, Phenol, Carbohydrate, Steroids, Protein and Resin. Although, when HPTLC study were carried out only Gallic acid was found which may have potent anticancer activity. In in-vitro study, At 500 µg/ml ($P<0.001$) and 1000 µg/ml ($P<0.001$) the Optical Density was found to be significantly decreased as compared to PC. There is a significant difference in mean among the seven groups. The Cell viability MTT assay also shows significant growth inhibition, at the same time statistical analysis of MTT assay also proved significant results. At last we concluded that the CO_2 extract of fruit of *Terminalia chebula* has shown significant activity on Human breast cancer cell line MDA-MB-231.It is humbly submitted that this study might guide to forthcoming researchers to establish standard for fruit of *Haritaki* for strict quality control and assurance. Inspiring pre-clinical results might serve as a ray of hope for further researchers to evaluate its efficacy with clinical trials. Also with the help of channelized research and innovations, Ayurveda will definitely stand out to be the ultimate weapon in the fight against dreadful disease like Cancer.

Limitations of the current study:

1. The present study was concentrated on evaluation of In-vitro anticancer activity using MTT assay protocol. If compound is not soluble in solvents, while screening, using MTT assay protocols, it was difficult to study pharmacokinetics of the study drug and to examine the activity.
2. It was difficult to maintain the cell culture even after providing specific conditions. Due to the limitation over dosage system and comparison of results with control drug i.e. DMSO these types of assays are inappropriate for screening of herbal extracts.

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None

CONFLICT OF INTEREST-

The authors have no conflicting financial interests.

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