

Efficacy of CO₂ extract of Vasa (*adhatoda vasica* linn.) leaves, in breast cancer cell line – MCF 7, for its cytotoxic effects

DR. ASHWINI MAKADI^[1], DR. PANKAJ BARAPATRE ^[2]

- 1. Assistant professor, Department of Dravya Guna, Sardar Patel Ayurvedic College & Medical Hospital**
- 2. Assistant professor, Department of Shalayatantra, Sardar Patel Ayurvedic College & Medical Hospital**

Abstract

Cancer is a complex disease in which there is an abnormal growth of cells in the body, which leads to death. These cells are born due to an imbalance in the cell proliferation mechanism. Breast cancer is the most common invasive cancer in women worldwide, and it is also the leading cause of death from cancer among women. In India, breast cancer is the leading cause of cancer deaths in women, and the risk of its incidence is increasing every year. Ayurveda is one of the world's oldest medical systems. It relies on a natural and holistic approach to physical, mental, and emotional health. It is known from very early times for preventing or suppressing various tumors using various natural drugs.

Vasa (Adhatoda vasica Nees.) is a well-known herbal drug in the Ayurvedic System of Medicine. A drug known as *Vasa or Vasaka* consists of fresh dried leaves and roots of *Adhatoda vasica*. This plant is a source of Vitamin C and has medicinal uses, mainly antispasmodic, antipyretic, antitussive, anti-inflammatory, bronchodilator, and oxytocic. It is astringent, diuretic, anti-periodic, purgative, and expectorant. There are several studies done to show the antitumor, antioxidant, anti-inflammatory, anti-mutagenic, and radioprotective activity of *Vasa* on different cancer cell lines. So, by using this anti-tumor property of *vasa* and to prove the efficacy of Ayurvedic herbs as an alternative and cost-effective source of treatment of cancer, this study has been undertaken for an in vitro study on the breast cancer cell line MF-7 by using CO₂ extract of *Adhatoda vasica* leaves using MTT assay.

Keywords: Breast cancer; cytotoxic effect; *vasa (adhatoda vasica* Linn.) In-vitro; MCF-7 Cell-line, co₂ extract

1. Introduction

Cancer is a serious problem affecting the health of all human societies. Cancer burden in India has more than doubled over the last 20 years. After cardiovascular diseases, cancer is the second most common disease for the maximum deaths in the world. [1]

Breast cancer is the most common cancer diagnosed among women in the U.S. Each year, about 32% of all newly diagnosed cancers in women are breast cancer. In 2025, approximately 316,950 women will be diagnosed with invasive breast cancer, with 59,080 new cases of ductal carcinoma in situ (DCIS), which is non-invasive. About 16% of women with breast cancer are younger than 50 years of age. About 66% of breast cancer cases are diagnosed at a localized stage[2]

As for breast cancer, MCF-7 cells represent a very important candidate as they are used ubiquitously in research for estrogen receptor-positive breast cancer cell experiments, and many subclones that have been established represent different classes of ER-positive tumors with varying nuclear receptor expression levels.[3]

In Ayurveda, cancer-like conditions are described under concepts such as: *Arbuda* (major tumors) and *Granthi* (minor tumors) Cancer is viewed as an imbalance of: *Doshas* (*Vata*, *Pitta*, *Kapha*) *Dhatus* (body tissues) *Agni* (digestive/metabolic fire) *Ojas* (immunity and vitality) Nowadays research is going to invent an Ayurvedic medicine to prevent or to cure cancer; vasa (*Adhatoda vasica*.) is one of the most important drug. This plant contains alkaloids, tannins, flavonoids, terpenes, sugars, and glucosides. The principal constituents of *Vasa* are its several alkaloids, the chief one being vasicine, which possesses the anti-cancer property.[4]

According to *Bhavprakash Vasa*, *Adhatoda vasica* is an Ayurvedic drug of the Acanthaceae family. It rasapanchak includes *Katu*, *Tikta Rasa with Katu Vipaka*, *Sheeta virya*, and *laghu, ruksha* guna, which is useful in the treatment of *Apachi*, *Gulma*, and *Ganda* [5,6]. By considering all the properties of the *Adhatoda vasica* Drug, here we took the CO₂ extract of leaves of the vasa plant and the MCF-7 breast cancer cell line. This study was done by using the MTT assay to determine cell viability and cell toxicity.

Aim and objectives

To Study of Cytotoxic and apoptotic effects of CO₂ extract of Vasa (*Adhatoda vasica*) leaves, in MCF:7 breast cancer cell line.

2. Materials and methods

The present study was carried out under the following headings.

2.1. Section I

2.1.1. Collection of sample

The botanically identified sample of Vasa (*Adhatoda vasica*) leaves was collected from the local area and other parts of the plant, such as pods, flowers, and stems, for further medicinal uses.

2.1.2. Authentication

The authenticity of this sample was confirmed by comparing its characters with various floras by a botanist from an authorized institute.

Pharmacognostical, Physicochemical, and Phytochemical study^[7]

It includes

- Raw material standardization
- In process standardization
- Finished product standardization

2.2. Pharmacognostical study

The parameters selected for the pharmacognostical study were as follows:

2.2.1. Part 1. Raw material standardization

Macroscopy of Vasa (*Adhatoda vasica*) leaves ^[8]

Procedure

The external features of the test sample of Vasa (*Adhatoda vasica*) leaves were examined by the naked eye, using a magnifying lens.

Figure 1 – vasa leaves

Table 1 Organoleptic evaluation of Vasa (*Adhatoda vasica*) leaves^[9]

Sr. No	Characters	Observation
1	Colour	Green – brown
2	Odour	Characteristic strong odour
3	Taste	Bitter, pungent
4	Shape	Ovate – lanceolate
5	Size	10-13 cm long
6	Surface	Glabrous and hairy
7	Texture	Leathery

2.3. In process drug standardization ^[10]

Physicochemical analysis: The air-dried leaves of Vasa (*Adhatoda vasica*) were powdered finely and subjected to various analyses, such as determination of foreign matter, moisture content, ash value, acid-insoluble ash, water-soluble ash, etc. The extractive value in various solvents and the ash value are important in the identification and standardization of a single drug.

Table 2 Physicochemical parameters ^[11]

SR.NO	Test	API Standard value	Powder of vasa leaves
1	Loss on drying	-	4.31
2	Total Ash	Not more than 21 %	14.34
3	Acid – insoluble Ash	Not more than 1%	0.86
4	Alcohol – soluble extraction	Not more than 3%	0.95
5	Water- soluble extraction	Not more than 22%	18.36
6	Ph		8.32

2.4. Finished product standardization

2.4.1. Preparation of CO₂ extract of vasa leaves [12]



Figure 2: Vasa leaves powder, Figure 2: CO₂ extraction machine

Figure 4- CO₂ extract of Vasa leaves

Table 3 Finished product standardization

Sr. no	Tests	CO ₂ extract of leaves of Vasa
1	pH	5.71
2	Specific gravity	1.2163
3	Total solids	57.74%

Table 4 Heavy Metal Tests

	Result	Permissible limits
Lead	< 1 ppm	Not more than 3 ppm
Arsenic	< 5 ppm	Not more than 1.0 ppm
Mercury	< 2 ppb	Not more than 0.1 ppm

Cadmium	<0.1 ppm	Not more than 0.3 ppm
---------	----------	-----------------------

Table 5 Microbial contamination ^[13]

	Limits	Results
Total Plate Count	Not More Than 10 ⁵ cfu/gm	30 cfu/gm
Total Yeast and Mold Count	Not More Than 10 ⁴ cfu/gm	<10 cfu/gm
Salmonella	Should be absent in 25 gm	Absent
E . coli	Should be absent in 1 gm	Absent
Enterobacteria	Not More Than 10 ⁴ cfu/gm	Absent

Table 6 Phytochemical analysis of CO₂ extract of Adhatoda vasica leaves

Phytochemical test	Result
Carbohydrate 1. Fehling Benedicts	Present
Alkaloids 1. Wagners 2. Mayer's	Present
Glycoside 1. Legal test	Present
Tannin 1. Gelatin 2. FeCl ₃	Absent
Flavonoids 1. alkaline reagent	Present
Protein 1. Biurette test	Absent
Saponin 1. Froath test	Absent

2.4.2. HPTLC^[14]

HPTLC is used for the identification of constituents, the identification and determination of impurities, and the quantitative determination of active substances.

Application Mode	CAMAG Linomat-5 Applicator
Filtering System	Whatman filter paper No.1
Stationary Phase	MERCK-TLC/HPTLC Silica gel 60 F ₂₅₄ on Aluminum sheets
Application (Y axis) Start Position	10mm
Development End position	80mm from the plate base
Sample Application Volume	<i>Vasa</i> (CO ₂ extract of <i>Adhatoda vasica</i>), 3.0µL
Distance Between Tracks	20mm
Development Mode	CAMAG TLC Twin Trough Chamber
Chamber Saturation Mode	30 minutes
Mobile Phase	Toluene: Ethyl acetate: Acetic acid (7: 2: 1 v/v)
Visualization	@254nm, @366nm (after derivatization) and @540 nm (after derivatization)
Spray Reagent	Anisaldehyde- sulphuric acid reagent
Derivatization Mode	CAMAG -Dip tank for about 1 minute
Drying Mode, Temp. & Time	TLC Plate Heater Preheated at 100±5°C for 3 minutes

HPTLC plate of CO₂ extract of leaves of *Adhatoda vasica* scanned at 254 wavelength showed the presence of ten phytochemical constituents value 0.14 to 0.85, in which component no. 5 is present in the highest amount, with R_f value ranging from R_f 0.59

2.4. Section II

In vitro study (assessment of anti-cancer activity of *Adhatoda vasica*)

Table 8: Cell line of breast cancer used for the study

Sr. no	Cell line	Tissue of an organ
1	MCF-7	Breast cancer

2.5.1. Method of in vitro anti-cancer study

The cytotoxicity is checked by using the MTT assay. The general purpose of the MTT assay is to measure viable cells in relatively high throughput (96-well plates) without the need for elaborate cell counting.

2.5.2. Cancer cell line culture

Day 1

- 104 cells/well were 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% CO₂ incubator for 24 hours

. Day 2

- After 24 hours of incubation, the plate was observed under an 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 was added in triplicate 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% CO₂ incubator for 24 hours.

Day 3

- After 24 hours of 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.

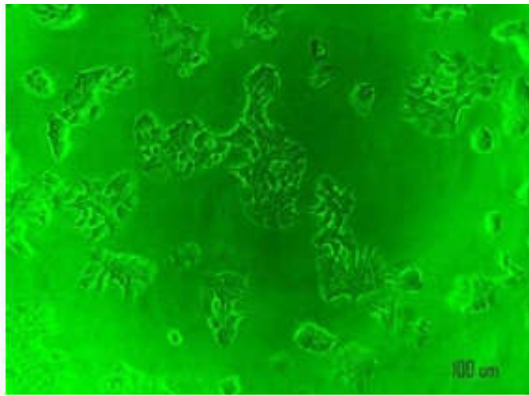
- Then 10µl of MTT reagent was added to each well.

- The plate was wrapped in aluminium foil and incubated at 37 °C in a 5% CO₂ incubator for 4 hours.

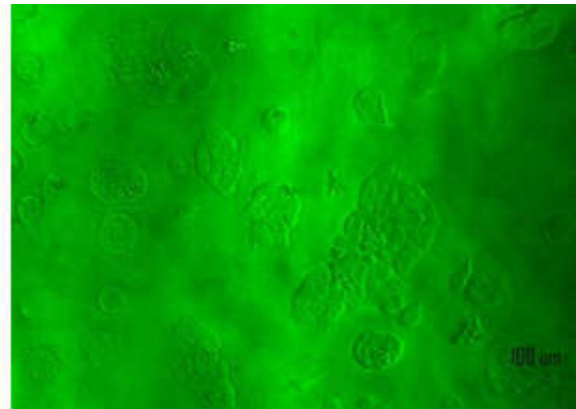
- Post 4 hours incubation, the entire medium was removed by flicking the plate, and 100 µl of solubilisation buffer was added to each well and incubated at 37°C in a 5% CO₂ incubator for about 20 minutes. 6. Post incubation, absorbance was measured at 570nm and 630 nm on a 96-well plate reader.

Table 9 Observation of Drug Concentration with O.D

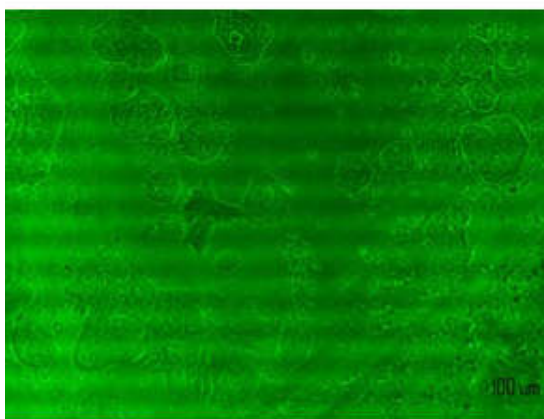
Sr.No	Drug Concentration $\mu\text{g/ml}$	Average O.D values
1	C	0.234
2	Positive control	0.134
3	40 $\mu\text{g/ml}$	0.216
4	60 $\mu\text{g/ml}$	0.195
5	100 $\mu\text{g/ml}$	0.172
6	120 $\mu\text{g/ml}$	0.154
7	250 $\mu\text{g/ml}$	0.145
8	500 $\mu\text{g/ml}$	0.132



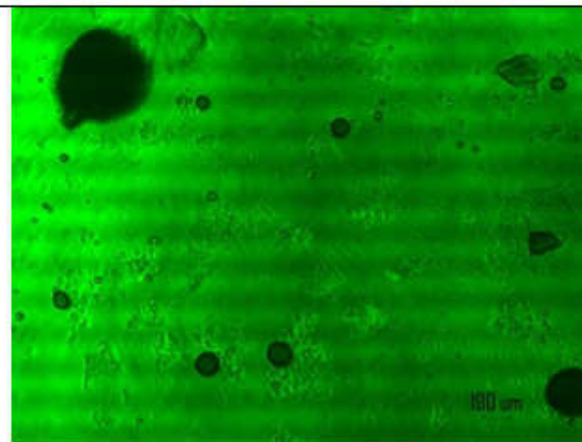
MCF-7 Control



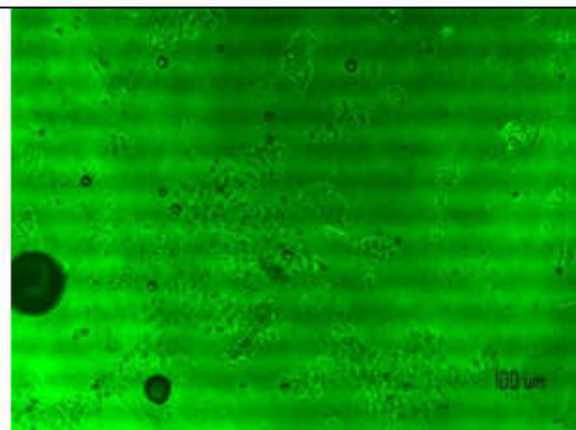
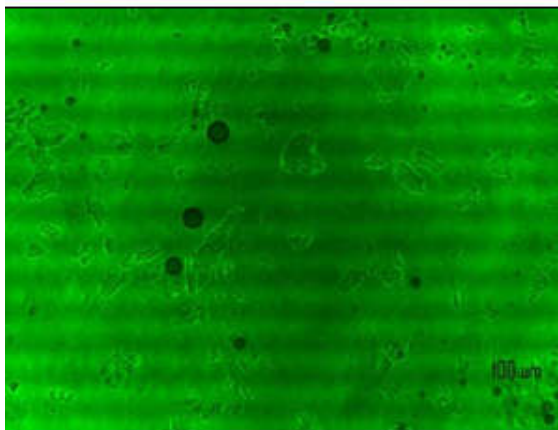
MCF-7 Positive control



MCF-7 40ug/ml



MCF-7 60ug/ml



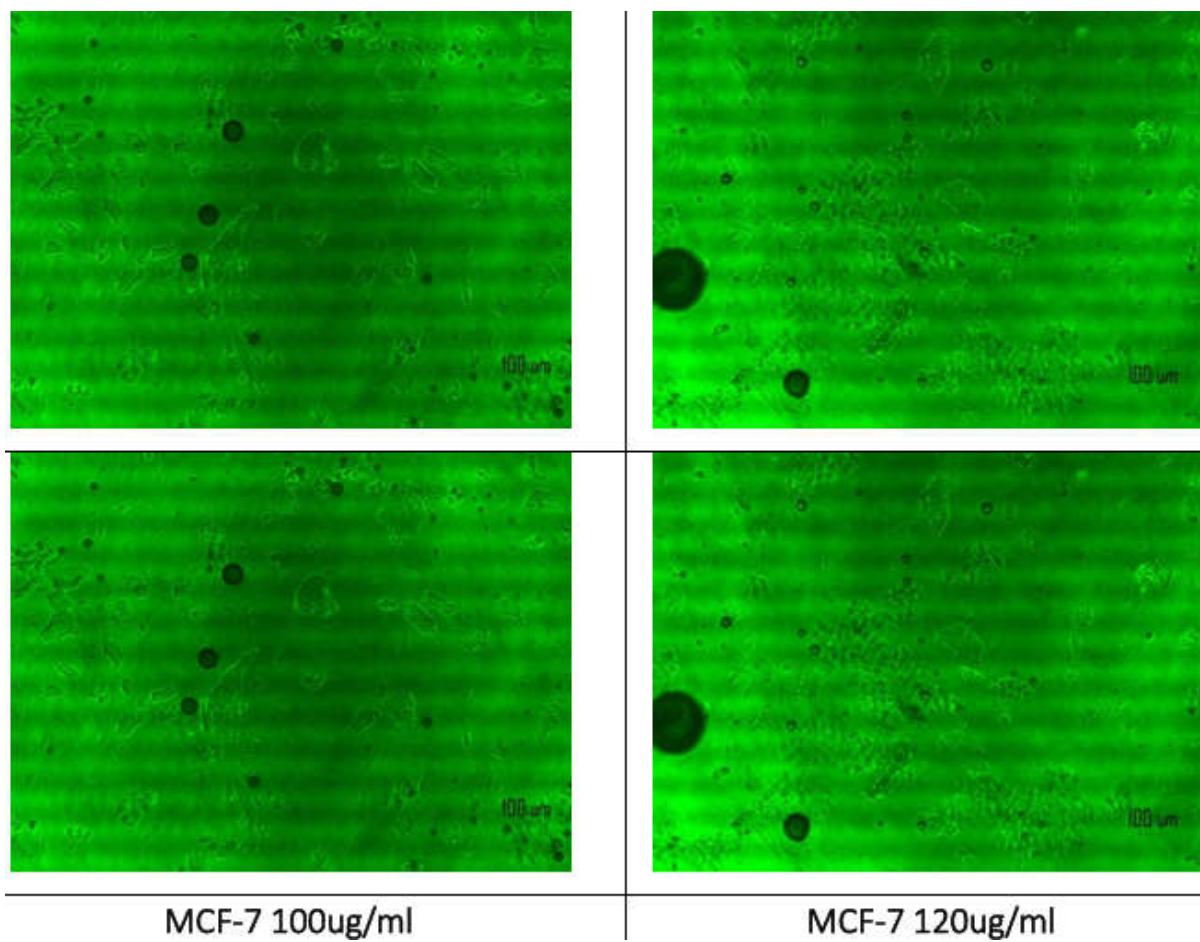


Figure 1: Optical density at different concentrations on MCF-7

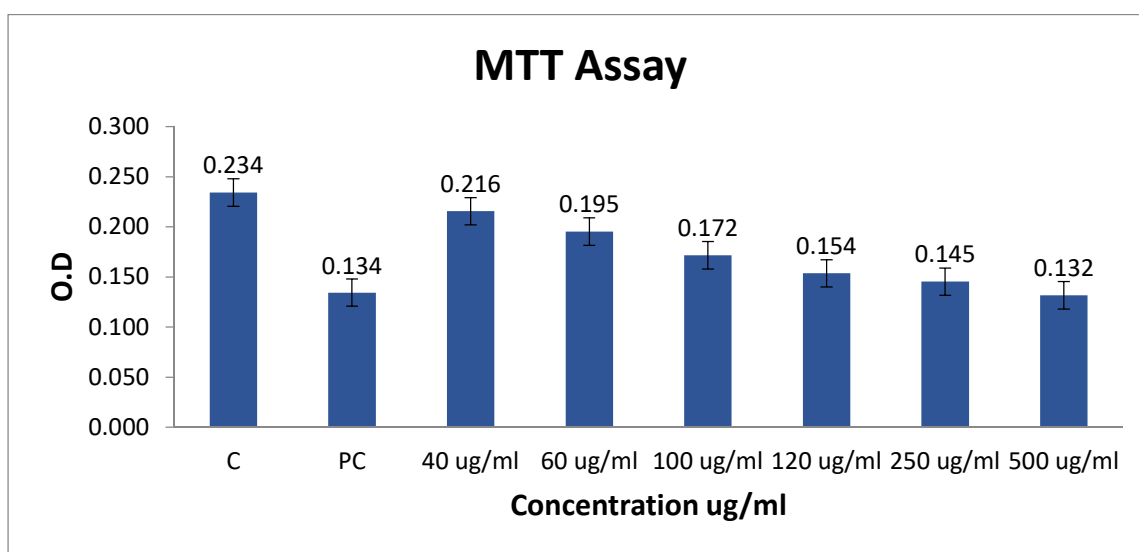


Figure 2 Graphical description of Optical density at different concentrations on MCF:7

Observation:

As the concentration increases, cytotoxic activity increases, and as the concentration decreases, cytotoxic activity decreases. It suggests that cytotoxic activity is dependent on the concentration of the drug. So, the cytotoxicity is dose-dependent. The highest toxicity was seen at the concentration of 500 $\mu\text{g/ml}$ as compared to the positive control. Whereas the remaining concentrations, i.e., 40 $\mu\text{g/ml}$, 60 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 120 $\mu\text{g/ml}$, and 250 $\mu\text{g/ml}$, have shown less toxicity as compared to the positive control.

Cytotoxicity of the sample was carried out on MCF:7 cell lines by using the MTT assay. As compared to C (control group), the optical density (OD) of PC (positive control) was significantly ($P<0.001$) less, indicating significant cell death. According to the result, it can be concluded that the increase in concentration of the CO₂ extract of vasa leaves shows a significant increase ($P<0.0001$) in cytotoxicity in the cell line as compared to the control group

3. Discussion

The test drug *Adhatoda vasica* proved standard by comparing with all API standard values with the help of various standard tests such as Pharmacognostical, Physicochemical, phytochemical, Heavy metal test, and Microbial contamination.

As per the results obtained from the MCF 7 breast cancer cell line, the concentration of the study was increased up to 500 $\mu\text{g/ml}$. DMSO was used as the control group. Graphical representation was done by plotting a bar diagram on the basis of the average value obtained from each experiment, which denotes the effect of the drug and the control drug on the selected cancer cell line.

The results can be discussed for an anticancer study on breast cancer cell line MCF 7 were as follows: Statistical analysis of various concentrations from 60 $\mu\text{g/ml}$ to 500 $\mu\text{g/ml}$ shows significant anti- cancerous action, i.e., cytotoxic activity on MCF 7 breast cancer cell lines. The study drug was taken in six concentrations, i.e., 40 $\mu\text{g/ml}$, 60 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 120 $\mu\text{g/ml}$, 250 $\mu\text{g/ml}$, and 500 $\mu\text{g/ml}$. Each was compared to the Standard drug (DMSO).

The highest toxicity was seen at the concentration of 500 $\mu\text{g/ml}$ as compared to the positive control. Whereas the remaining concentrations, i.e., 250 $\mu\text{g/ml}$, 120 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 60 $\mu\text{g/ml}$, and 40 $\mu\text{g/ml}$, have shown less toxicity as compared to the positive

control. As per the above obtained results, it can be stated that as the concentration increases, cytotoxic activity increases, and as the concentration decreases, cytotoxic activity decreases. This suggests that cytotoxic activity is dependent on the concentration of our drug. So, we can conclude that cytotoxicity is dose-dependent. And the drug has shown positive results at a 500ug/ml concentration.

4. Conclusion

- The study was carried out by using all standard protocols to test the drug *Adhatoda vasica*. The efficacy of vasa was checked by doing all standard parameter tests, such as Pharmacognostical, Physicochemical, phytochemical, Heavy metal test, Microbial contamination, and the drug was proven by all API standard values.
- Cytotoxicity of the sample was carried out on MCF:7 cell lines by using MTT assay. According to the results, it can be concluded that the increase in concentration of the CO₂ extract of vasa (*Adhatoda vasica*) leaves shows an increase in cytotoxicity in the MCF-7 cell line.

Further scope and Limitations of the study

CO₂ extract of vasa (*Adhatoda vasica*) leaves. has shown promising anticancer potential. The following are the future scopes of the current research to develop these extracts as a drug.

- Standardization of the CO₂ extract of vasa (*Adhatoda vasica*) leaves. should be done with the help of more precise and advanced parameters of testing.
- Further study required to comparative study on different cell line for the efficacy and toxicity of the CO₂ extract of vasa (*Adhatoda vasica*) leaves in different cell lines.
- The study should be conducted using different extraction method like soxhlet extraction, decoction, percolation, etc.

Compliance with ethical standards

Acknowledgments

I am thankful to my co-authors for their support and guidance across the study. I am thankful to the Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University, for the authentication of *Adhatoda vasica*. Drug, also, I express my sincere regards and gratitude to the Managing Partner of Nisarga Biotech Private Limited. Satara for providing the pure CO₂ extract of leaves of *Adhatoda vasica*. I am also thankful to the director of Bhide Foundation,

Pune, for various physicochemical, Microscopic, Heavy metal analysis, etc. tests. And I express sincere regards to the APT Testing Research Private Limited, Pune, for giving me the great opportunity to conduct my study in their lab, and for providing MCF-7 cell lines. And I express sincere regards to the APT Testing Research Private Limited, Pune, for giving me the great opportunity to conduct my study in their lab, and for providing MCF-7 cell lines.

REFERENCES

- 1].WHO report on cancer: setting priorities, investing wisely and providing care for all. Geneva: World Health Organization; 2020. License: CC BY NC-SA 3.0 IGO.
- 2].Maurie Markman, MD, President, Medicine & Science at CTCA, cancer treatment, centers of America, types of breast cancer, updates, May 24, 2021
- 3].Breast cancer classification <http://en.m.wikipedia.org>
- 4].Narendra Kumar et al. Pharmaceutical attributes of Vasa- A Review, WJPR, 2016, vol (5), Issue 4, 437-453
- 5].Ram Manohar P, Description and classification of cancer in classical Ayurvedic texts, Ind. J. Sci. 2015 (cited 2017 Aug); 50(2); 187-195Taranath BhattAcharya. Shabdasatoma mahanidhi. 3rd edition Varanasi; Chaukhambha Sanskrit Series office. 1967; p.53.
- 6], Dr krishnachandra Chunekar, Bhavprakash Nighnatu, Guduchaydi varga 34\307-309, Chaukhamba Bharti Academy Varanasi, Chaukhamba Publication (2018)
- 7] Vasa(leave): The Ayurvedic Pharmacopeia of India; Government of India; Ministry of Health & Family Welfare; Department of Ayush. Part I vol (1) pg-122
- 8] K. R. Khandelwal and Dr. Vrunda Sethi. Practical Pharmacognosy. 26th edition. Pune; Nirali Prakashana. February 2016. p. 2.1-3.5
- 9] Dhale DA, Kalma RK, Pharmacognostic characterization of Adhatoda vasica medicine: International Journal of Pharmaceutical Science and Research: 2012,3(11), 4264-4269.
- .10] [A.P.I. Part 2, Vol 4, p.no. 140]
- 11] K. R. Khandelwal and Dr. Vrunda Sethi. Practical Pharmacognosy. 26th edition. Pune; Nirali Prakashana. February 2016. p. 20.1-20.5 197. Shriradhakrushna Parashar (Hindi

Translation). 5th edition. Nagpur; Shri Vaidyanath Ayurved Bhavan Prakashana. August 2012. p.238

12] S. Sasidharan. Y. Chen. D.Saravanan. K.M. Sundaram. L.Yoga Latha. "Extraction, isolation, and characterization of bioactive compounds from plant extracts." Afr J Tradit Complement Altern Med. 2011;8(1):1-10167

13] Anonymous. The Ayurvedic Pharmacopoeia of India (Part II, vol 1, Appendix 2.4), 1st edition. 1990, Reprinted 2001. p.163

14] Sudberg S, Terrazas J et al. Fingerprint analysis and the application of HPTLC to the determination of identity and quality of botanicals, AOAC Int. 2010; 93: 1367-75